CHEMOKINE ORCHESTRATION OF LEUKOCYTE-SKIN INTERACTIONS

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Psoriasis is a common disease, characterised by thick erythematous skin plaques and scaling, as well as a range of associated conditions, including psoriatic arthritis. Psoriatic skin lesions are characterised by excessive inflammation, leukocyte infiltration and production of chemokines, the primary regulators of leukocyte migration. The chemokines that drive immune cell recruitment to the lesions, sequence of migratory events, and drug effects on leukocyte migration remain poorly understood. Therefore, we have investigated the chemokine-orchestrated leukocyte migration events in psoriasis and how they might be affected by current in-use drugs for the treatment of psoriasis. Using a transwell system, which assays cell chemotaxis towards a specific chemoattractant, we demonstrate that CXCL8 and CCL20 are important drivers of lesion recruitment of neutrophils and T-cells respectively in psoriasis. Furthermore, neutrophils and T-cells derived from patients with psoriasis had an increased responsiveness to CXCL8 and CCL20, respectively. Additioally, we also demonstrate that the increase in CXCL8 responsiveness is diminished in patients treated with the PDE4 inhibitor Apremilast. Strikingly, Apremilast treatment of neutrophils led to the abolishment of intracellular calcium fluxes, known to be related to reduced neutrophil migration. Further to this, we show that Apremilast treatment of mice with psoriasiform inflammation significantly reduced neutrophil influx into the lesions. Interactions of leukocytes with keratinocytes have been previously linked to the elevated production of multiple pro-inflammatory mediators. Here we report that CCL20-responsive T-cells, derived from patients with psoriasis, were able to induce the production of neutrophil attracting factors by keratinocytes. On the contrary, CXCL8-responsive neutrophils, derived from patients with psoriasis, were not able to induce the production of T-cell attracting factors by keratinocytes. The data suggest that immune-stromal cell interactions can influence immune cell recruitment in psoriasis, but more work is required to determine whether or not this is a fundamental pathway that can be targeted.

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Abbreviations

µM: Micro molar

AA: Amino acid

AC: Adenylyl cyclase

ACKR: Atypical chemokine receptor

AKAPs: A-kinase-anchoring proteins

AMPs: Antimicrobial peptides

APC: Allophycocyanin

APCs: Antigen presenting cells

ATP: Adenosine triphosphate

Bcl-6: B-cell lymphoma 6 protein

Blimp-1: B lymphocyte-induced maturation protein-1

BM: Bone marrow

BP: Base-pair

BSA: Bovine serum albumin

C57BL/6J: C57 black 6

Ca^{2+}: Calcium ions

cAMP: Adenosine 3',5'-cyclic monophosphate

CCL: chemokine ligand (CC-type)

CD: Cluster of differentiation

cDC: Conventional dendritic cell

cDNA: Complementary DNA

CGD: Chronic granulomatous disease

CKR: Chemokine receptor

CLA: Cutaneous lymphocyte-associated antigen

CLR: C-type lectin receptors

CMC: Carboxymethyl cellulose

CREB: cAMP-response element-binding protein

CTLA: Cytotoxic T lymphocyte antigen

CXCL: chemokine ligand (CXC type)

DAG: Diacylglycerol

DAMP: Danger-associated molecular patterns

DC: Dendritic cell

DLQI: Dermatology Life Quality Index

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

dNTPs: Deoxynucleoside triphosphates

DP: Double positive

EBM: Endothelial basement membrane

EC: Endothelial cell

EPAC: Exchange protein directly activated by cAMP

ER: Endoplasmic reticulum

FACS: Fluorescence-activated cell sorting

FBS: Fetal Bovine serum

FCS: Foetal calf serum

FITC: Fluorescein

FOXP3: Forkhead box P3

GAGs: Glycosaminoglycans

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

G-CSF: Granulocyte colony stimulating factor

GDP: Guanosine diphosphate

GPCR: G-protein-coupled receptor

GPP: Generalised pustular psoriasis

GRKs: G-protein-coupled receptor kinases

GTP: Guanosine triphosphate

GWAS: Genome-wide association studies

H&E: Hematoxylin and eosin

HBSS: Hanks' Balanced Salt Solution

HEPES BSS: HEPES buffered Balanced Salt Solution

HIF: Hypoxia inducible factor

HLA: Human leukocyte antigen

HSC: Hematopoietic stem cell

iDCs: Inflammatory dendritic cell

IFN: Interferons

IL: Interleukin

IMQ: Imiquimod

IP3: Inositol trisphosphate

iTreg: Induced regulatory T-cell

JAK: Janus kinase

JAM: Junctional adhesion molecule

K: Thousand

K_3EDTA: K3 Ethylenediaminetetraacetic acid

KC: Keratinocyte

LBRC: Lateral border recycling compartment

LFA-1: Lymphocyte function-associated antigen 1

LGP-2: Laboratory of genetics and physiology-2

LN: Lymph node

LPS: Lipopolysaccharide

LRR: Leucin rich repeats

Ly6: Lymphocyte antigen 6

MAPK: Mitogen-activated proteins kinases

MDA5: Melanoma differentiation factor-5

mDC: Myeloid dendritic cell

Mg: Magnesium

MHC: Major Histocompatibility Complex

mL: Millilitre

MLCK: Myosin light-chain kinase

mPASI: Modified PASI

mRNA: Messenger Ribonucleic acid

MuLV: Murine Leukemia Virus

MYD88: Myeloid differentiation primary response gene 88

NADPH: Nicotinamide adenine dinucleotide phosphate

ND: Neutrophilic dermatoses

NETs: Neutrophil extracellular traps

NF: Nuclear factor

NHEK: Normal Human Epidermal Keratinocytes

NK: Natural killer cells

NLR: NOD-LRR-containing receptors

NOD: Nucleotide-binding oligomerization domain

NTC: Non-template control

nTreg: Natural regulatory T-cell

P3: Passage 3

PAD4: Peptidylarginine deiminase 4

PAM: Polyacrylamide

PAMP: Pathogen-associated molecular patterns

PASI: Psoriasis Area and Severity Index

PB: Peripheral blood

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate-buffered saline

PDAR: Psoriatic disease research

pDC: Plasmacytoid dendritic cell

PDE: Phosphodiesterase

PDE4: Phosphodiesterase 4

PE: Phycoerythrin

PECAM-1: Platelet endothelial cell adhesion molecule-1

PE-Cy7: Phycoerythrin-Cy7

PerCP: Peridinin-Chlorophyll-Protein

PI3K: Phosphoinositide 3-kinase

PIP3: Phosphatidylinositol (4,5)-trisphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PM: Plasma membrane

PMCA: Plasma membrane Ca^{2+} ATPase

PMN: Polymorphonuclear

PRR: pattern recognition receptors

PSGL1: P-selectin glycoprotein ligand 1

PSORS: psoriasis susceptibility

QoL: Quality of life

qPCR: Quantitative Polymerase Chain Reaction

RAR: Retinoic acid receptor

RDAR: Rheumatoid disease research

RIG1: Retinoic acid-inducible gene 1

RLR: RIG-1-like receptors

RNA: Ribonucleic acid

RNases: Ribonucleases

ROR: Retinoic acid receptor-related orphan receptor

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute

RT: Reverse transcription

S1P: Sphingosine 1-phosphate

S1P: Sphingosine 1-phosphate

sAC: Soluble ACs

SALT: Skin-Associated Lymphoid Tissue

SCID: Severe combined immunodeficiency

SEM: Standard error of the mean

SERCA: Sarco(endo)plasmic Ca^{2+} reticulum ATPase

SFRP4: Secreted frizzled-related protein 4

SOCE: Store-operated calcium entry

STIM1: Stromal interaction molecule 1

T-bet: T-box transcription factor

TBP: TATA-binding protein

TE buffer: Tris/EDTA buffer

TEM: Transendothelial migration

TF: Transcription factor

TFH: T follicular helper cell

Th: Helper T-cell

TIR: Toll/IL-1 receptor

TIRAP: TIR domain containing adaptor protein

TLR: Toll-like receptor

tMAC: Transmembrane ACs

TNF: Tumour necrosis factor

TNFR: TNF receptors

TNS: Trypsin Neutralization Solution

TPS: Transwell permeable support

TRAM: TRIF-related adaptor molecule

Treg: Regulatory T-cell

Trms: Tissue-resident memory T-cells

UV: Ultravilet

V450: Violet450

V500: Violet 500

VE: Vascular endothelial

VEGF: Vascular endothelial growth factor

Chapter 1: Introduction

1.1 Psoriasis

Psoriasis is a chronic, immune-mediated disease, which affects 1-3% of the worldwide population[1]. It has a profound impact on those affected in terms of morbidity and mortality. Psoriasis is characterised by the development of red skin plaques covered with silvery scale. Despite its characteristic skin lesions, there is increasing recognition that psoriasis is a systemic disorder, associated with significant co-morbidities (e.g. cardiac and metabolic disorders and psoriatic arthritis) [2-5]. Treatment of psoriasis is often complex, as it is done on a case-to-case basis and depends on disease severity. The better understanding of psoriasis pathogenesis and subsequent development of therapeutics has had a profound impact on disease prognosis.

1.1.1 Skin: Structure & Function

1.1.1.1 Health

The skin forms the outermost physical, immunological and biochemical barrier of the human body. The skin allows for the simultaneous maintenance of body homeostasis and a dynamic relationship with the external environment. This is possible due to the structure of the skin, which is composed of two main compartments: the dermis and the outermost epidermis. The epidermis is a continuously self-renewing layer and is usually further subdivided into several strata (Figure 1.1A). The building block and most predominant cell type of the epidermis is the keratinocyte. Keratinocytes (KCs) are viable and nucleated in the stratum basale, where about 10-15% of them are constantly mitotic[6]. The quiescent KCs in stratum basalis become proliferative only when necessary (e.g. wound healing)[7]. During differentiation, KCs are pushed towards the skin surface by newly formed cells. The maturation of KCs and their progress through strata: spinosum, granulosum, lucidum, and corneum is associated with changes in gene, protein and lipid expression[8,9, 10]. These changes are reflected by the structure and appearance of the different strata and are most drastic in stratum corneum. The stratum consists of flat, cornified, anucleated squamous KCs, which are filled with keratin filaments and surrounded by a protein-lipid envelope. The structure of the stratum corneum allows for protection against physical harm of lower, more sensitive, strata from e.g. dehydration; infection. Although the physical barrier function is mainly attributed to the stratum corneum, cell-to-cell junctions in the lower layers provide further stability to the skin. The entire stratum corneum is replaced by proliferating KCs during a period of 28 days in a process of desquamation. In addition to KCs, melanocytes (melanin-producing cells), leukocytes, Merkel cells and stem cells can also be found in the

epidermis[11]. The epidermis attaches to the dermis at the basement membrane by the binding of the stratum to the basal lamina (the upper layer of the basement membrane). The strength of this connection is enhanced by the dermal papillae, which are folds in the upper portion of the dermis. The dermis is a thick layer of connective tissue, which contains elastin and collagenous fibre-producing fibroblasts; blood and lymph vessels; sweat glands; nerves, and other structures.

1.1.1.2 Psoriasis

The importance of the balance between keratinocyte proliferation and desquamation is highlighted by the pathophysiology of inflammatory skin diseases such as psoriasis (Figure 1.1B). In psoriasis, the proliferation and maturation rate of keratinocytes is increased, and keratinocytes migrate from basal to cornified layers in only 4, as opposed to 28 days in health[12]. With hyperproliferation, the epidermis becomes acanthotic (thickened) and the rete ridges increase in length. The cornified layer retains cell nuclei, which leads to parakeratosis and subsequent scaling of the skin. Blood vessels become dilated, which accounts for skin redness, and allow for increased leukocyte infiltration[13]. Incoming leukocytes can acquire a resident phenotype and become retained within confined spaces in the epidermis[14,15]. Interactions between incoming leukocytes and keratinocytes allow the disease to propagate itself and become self-sustained[16].

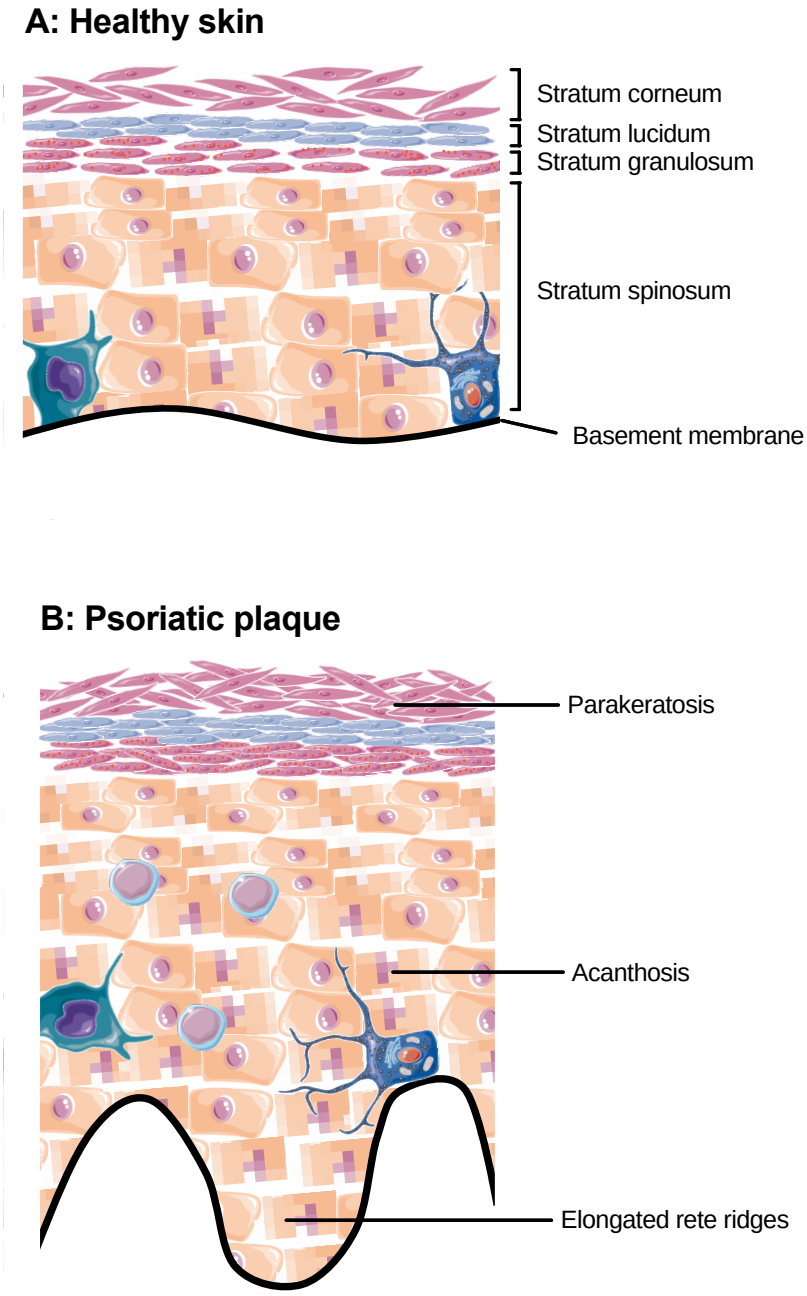


Figure 1.1 | Graphical representation of healthy skin and a psoriasis plaque

A) Diagram of healthy skin showing keratinocyte maturation through different epidermal strata

B) Diagram of a psoriasis plaque, showing acanthosis, elongated rete ridges, parakeratosis, lymphocyte infiltration

1.1.2 Diagnosis and clinical phenotypes of psoriasis

The clinical manifestation of psoriasis can be highly variable. Lesions can occur at any age; however, two peaks of incidence occurring at 20-30 and 50-60 years exist[17]. Furthermore, lesion presentation can differ dramatically between patients (e.g. lesions can take the form of plaques, pustules, papules, and more)[17]. Although the main disease manifestation is primarily associated with skin changes (which can be limited or extensive), it can also involve nails and joints[18]. The high variability has made the establishment of diagnostic criteria and classification of the clinical spectrum of cutaneous psoriasis a challenge.

1.1.2.1 Diagnosis of psoriasis

The diagnosis of psoriasis is primarily clinical. A clinical diagnosis is made via the examination of the skin lesion and its morphological properties[24]. The morphological features which reflect active skin lesions, include erythematous, silvery-white scaly, sharply demarcated plaques and are typically easy to recognise (Figure 1.2)[25]. However, where there is diagnostic uncertainty, a biopsy may be required. The timely diagnosis of psoriasis is extremely important to managing the disease and associated co-morbidities, which can often be of a psycho-social nature (due to social stigmatisation) [20]. Further to this, other co-morbidities that must be considered when diagnosing a patient with psoriasis include arthritis, metabolic syndrome, diabetes and hypertension [21-23]. As psoriasis is considered a systemic inflammatory condition, which can possibly affect multiple organs, patients require screening for co-morbid conditions (e.g. diabetes, arthritis) to improve long-term prognosis[26].

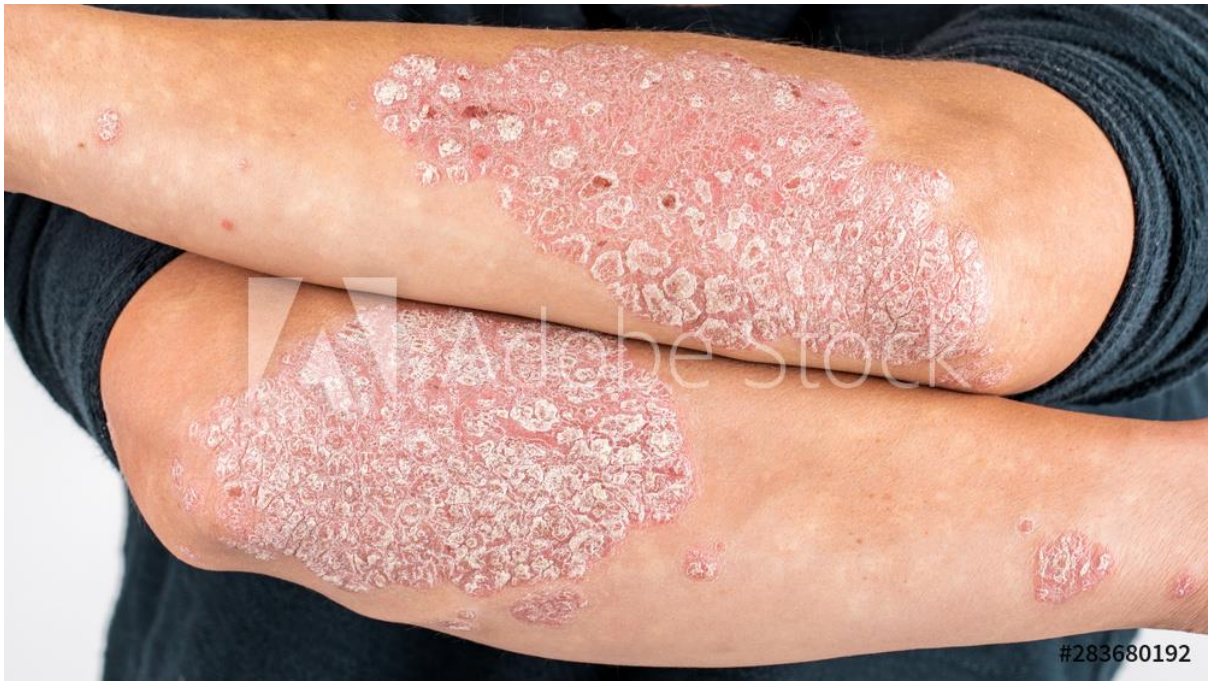


Figure 1.2| Image showing an active psoriasis lesion

The morphological features which reflect active skin lesions, include erythematous, silvery white scaly, sharply demarcated plaques, and are all shown on the image. Image taken from Adobe Stock images.

1.1.2.2 Phenotypes of psoriasis

Various features of psoriasis, such as morphology and anatomical site, have been used to classify the disease into different clinical subtypes[27-28]. This classification is clinically important, as treatment and prognosis between the phenotypes varies. The most common psoriasis subtypes, classified on the basis of morphology are psoriasis vulgaris, guttate psoriasis, pustular psoriasis, and erythrodermic psoriasis[29]. Scalp psoriasis, nail psoriasis, and palmoplantar psoriasis are classifications on the basis of involvement of specific anatomical locations. Psoriasis subtypes classified on the basis of anatomical locations are believed to be manifestations of morphological psoriasis variants.

Psoriasis vulgaris (or plaque psoriasis) is the most common form of psoriasis and represents almost 90% of all psoriatic patients[30]. The lesions usually begin as papules or macules which extend peripherally to form scaly, sharply demarcated round plaques. Although plaques are generally distributed symmetrically over extensor surfaces, such as elbows and knees, they can affect any body site (e.g. scalp, genital area, buttocks)[31]. New lesions can, in some, occur as a result of cutaneous traumaand generally remain until treated with low rates of spontaneous remission[32].

Guttate psoriasis accounts for ~3% of psoriasis cases and usually affects children and adolescents following a streptococcal or an upper respiratory tract infection[33-35]. The lesions appear as drop-like ('gutta') plaques, which are at the same stage of evolution and appear acutely. Although the disease is generally self-limiting and responds well to treatment, patients who have made a full recovery, are at a higher risk of developing plaque psoriasis in the future[36,37].

Pustular psoriasis is a rare form of psoriasis and is characterised by sterile, neutrophilic pustules with an underlying erythematous base[40]. Several types of pustular psoriasis have been recognised, the most severe of which is generalised pustular psoriasis (GPP)[38-39]. GPP is considered an active and unstable disease, the acute stages of which are associated with systemic symptoms, such as fever and myalgia[38-39, 41]. This is a life-threatening condition, which requires hospitalisation of patients. Although GPP has been considered a variant of pustular psoriasis, genetic studies, focussing on IL36RN gene mutations, have suggested that GPP is a different psoriasis phenotype[42-43]. Similarly, to GPP, Erythrodermic psoriasis is a life-threatening condition, which involves erythema of the majority of the body surface (>90%), and that can

lead to cardiovascular collapse and death in extreme cases. It is believed that these conditions may be precipitated by an infection or abrupt withdrawal of systemic corticosteroids[44-46].

Although the current diagnosis of psoriasis is primarily based on morphology, recent gene expression studies suggest a molecular heterogeneity in psoriasis subtypes[47,49]. For example, subtype-specific enrichment for signalling pathways, such as the TGFβ pathway, has been noted in plaque psoriasis[47]. Further to this, psoriasis subtypes have been shown to display differences in cytokine production profile[48]. The better understanding of psoriasis molecular signature could change methods of disease diagnosis and allow for a more precise, subtype-specific treatment of psoriasis.

1.1.3 Assessing psoriasis severity

The global assessment of the effects of psoriasis disease activity on patients' quality of life (QoL) and the physical severity are both used to determine disease severity and treatment success in patients. In the clinic, the gold standard for determining psoriasis severity is Psoriasis Area and Severity Index (PASI)[50]. The PASI score takes into account the basic characteristics of psoriasis: average redness, thickness, and scaliness of the lesions (each graded on a 0–4 scale), weighted by the area of involvement. This yields a score which helps categorise patients as mild (<10), moderate (10-15), or severe (15<). Although the PASI score is well-established and useful in quantifying disease severity (and is a regulatory requirement for prescription of biologic agents in the UK, that requires a PASI score of 10 or more), it has a number of limitations. Such limitations include a degree of subjectivity, inability to detect changes in small areas of involvement and poor correlation to QoL. Measurements of disease severity are an important inclusion criteria of patients in randomized controlled clinical trials. This is because an important trial outcome is the assessment of the drug efficacy in treating psoriasis. This requires a predetermined endpoint, which will demonstrate if patients can achieve clinically meaningful success when treated with the drug rather than placebo[51]. For example, patients with psoriasis must show at least 75% improvement in disease, as measured by the PASI scoring system (i.e. 75% improvement of PASI score, or 'PASI75)), at the study primary endpoint (e.g. 16 weeks). However, due to the poor ability of PASI to reflect QoL, determining clinically meaningful success has been challenged[52]. For example, it has been suggested that some patients that see lower improvement of PASI score can also have a clinically meaningful success in their disease[52]. QoL questionnaires, such as the Dermatology Life Quality Index (DLQI) and the Skindex, have been used alongside PASI in part to overcome some limitations of severity scores such as PASI[53,54].

DLQI takes into account symptoms and feelings, daily activities, leisure, work and school, personal relationships, and bother with psoriasis treatment. The resultant DLQI score can then be used to confirm if PASI score changes are indeed clinically meaningful. Furthermore, the development of new highly efficacious therapies may lead to an increase in the set predetermined endpoint, required for determining clinically meaningful success, and PASI90 and PASI100 as well as absolute PASI scores are now frequently reported[87a]. Additionally, in territories such as North America, the Psychian's Global Assessment (PGA) is frequently used in addition to/instead of the PASI score[87a]. Nevertheless, a standardised comprehensive approach to measuring disease severity is needed in order to establish best treatment course and develop future therapies.

1.2 Treatment of psoriasis

There is no cure for psoriasis, but many effective treatment options exist, and near clearance is now achievable in the majority of patients with newer biologic agents[88a]. The main treatment objectives are to limit disease severity and improve quality of life with a minimal treatment toxicity. Treatments include topical therapy, phototherapy, systemic and biological agents (Table 1.1). Treatment choice is generally made on an individual patient-to-patient basis and depends on disease severity at presentation and a multitude of other disease characteristics[55, 55a]. Mild to moderate psoriasis is usually treated with topical (direct on skin) agents, such as corticosteroids or Vitamin D3 analogues[57]. Topical treatment can be beneficial to a lot of patients and is good as an initial therapy for symptom control; however, adherence to therapy regimen is usually poor[56]. If topical agents are not effective in managing the disease or application is compromised by location, systemic therapy may be prescribed. Alternatively, ultraviolet light therapy is commonly used and can be highly effective but is not appropriate for all body sites (e.g. scalp). Systemic therapy (e.g. methotrexate) and systemic small molecule inhibitors (e.g. Apremilast) can be used for the treatment of moderate to severe psoriasis[58-60]. Patients that do not respond to systemic treatment or experience severe adverse effects, can be treated by biologic therapies, (where regulatory requirement are met[55a]) which work by blocking molecular mechanisms in psoriasis pathogenesis. The pathogenesis of psoriasis is complex and often patients do not respond to monotherapies, therefore, a combination of therapies can be prescribed[61]. Furthermore, treatment of psoriasis needs to be in accordance with existing co-morbidities, patient needs and collaborations with specialists may be necessary (e.g. if arthritis is present, a rheumatologist could typically become involved).

Table 1.1| Therapeutic agents used for treatment of psoriasis, their targets and mode of action.

Type	Agent	Target	Mode of action	Ref.
Topical therapy	Hydrocortisone (corticosteroid)	Glucocorticoid receptor (GCR)	GCR binding leads to inhibition of inflammatory transcription factors (e.g. NF-kappa B); the promotion of anti-inflammatory genes (e.g. IL-10)	62, 63
	Calcipotriol	Vitamin D receptor (VDR)	VDR binding believed to mediated expression of genes related to T-cell differentiation and proliferation	64, 65
	Anthralin	Molecular targets are unknown, but affects mitochondria, cellular enzymes, nucleic acid metabolism	Inhibition of keratinocyte proliferation via reduction of mitotic activity.	66
	Coal tar	Unknown	Unknown	67
	Pimecrolimus	Macrophilin-12	Calcineurin inhibition leads to T-cell and cytokine production inhibition	68,69
Systemic	Acitretin	Believed to target retinoid receptors in the skin	Inhibition of excessive cell proliferation, hyperplasia and keratinisation	70, 71
	Ciclosporin	Cyclophilin-1 inside Lymphocytes (T cells)	Inhibition of cytokines critical for lymphocyte proliferation	72

	Methotrexate	Dihydrofolate reductase (halts cell division)	Limits epithelial hyperplasia; apoptosis of activated T cells; inhibits the chemotaxis of neutrophils	73
	Dimethyl fumarate	Nrf2 (activation of)	Unknown	74
Phototherapy	Narrow band UVB (311nm)	Deoxyribonucleic acid (DNA)	Believed to induce apoptosis of leukocytes and keratinocytes	75, 76
	Psoralen UVA (325nm)	DNA	Believed to induce apoptosis of leukocytes and keratinocytes	77
Small molecule inhibitors	Apremilast	PDE-4	Inhibits PDE-4, and production of several cytokines e.g. TNF, IL-2, IFN	78
	Tofacitinib	JAK-1/ JAK-3	Disrupts the JAK/STAT signalling pathway and cytokine responsiveness	79, 80
Biologics	Certolizumab	TNF	Targets TNF	81
	Etanercept	TNF	Targets TNF	82
	Adalimumab		Targets TNF	83
	Infliximab	TNF	Targets TNF	84
	Ustekinumab	IL-12 and IL-23	Binds to cytokine to prevent receptor interactions and subsequent T cell activation.	85
	Tildrakizumab	IL-23 (p19 subunit)	Binds to cytokine to prevent receptor	86

			interactions and subsequent Th17 cell activation/maintenance.	
	Risankizumab	IL-23 (p19 subunit)	As above	87
	Guselkumab	IL-23 (p19 subunit)	As above	88
	Ixekizumab	IL-17A	Binds to cytokine to prevent receptor interactions and subsequent KC activation.	89
	Secukinumab	IL-17A		90
	Brodalumab	IL-17 receptor	Inhibits several pro-inflammatory cytokines from the IL-17 family (IL17A/F)	91

Original table

1.2.1 Topical therapy

Mild psoriasis is usually treated with topical (directly on skin) therapy. The most widely used topical agents that have shown therapeutic activity and are licensed for use are corticosteroids[63]. Topical steroids can have different strengths and formulations and are categorised into four different classes on the basis of their potency (low-, moderate-, high-; ultra-high-potency)[92]. Low-potency corticosteroids are used for the treatment of sensitive body areas where the skin tends to be thinner, such as face and genitals. On the other hand, ultra-high-potency corticosteroids are used in the treatment of very scaly lesions, such as those found on the scalp[92]. Topical corticosteroids act by inducing the transcription of anti-inflammatory genes (e.g. IL-10) and decreasing the transcription of pro-inflammatory genes (e.g. IL-1, TNF, CXCL8)[63]. Corticosteroid agents can also slow-down keratinocyte proliferation rates, cause vasoconstriction and lead to apoptosis of pro-inflammatory cells[63]. This in combination with their fast molecular action, makes them a preferred choice as a first-line of treatment in the management of psoriasis. However, the long-term use of topical corticosteroids can lead to adverse effects, such as skin changes (e.g. thinning of the skin) and tachyphylaxis[93]. Critically also, potent topical steroids alone can destabilise and worsen psoriasis.

1.2.2 Phototherapy and systemic treatments

Phototherapy is a common treatment and management of moderate to severe psoriasis, often unresponsive to topical agents. Phototherapy relies on ultraviolet (UV) light and is available at different emission spectrums (e.g. Narrow band ultraviolet B light has an emission spectrum of 311-313nm). The exact mechanisms of action are unknown, but the absorption of UV by nuclear DNA leads to cell cycle progression arrest and subsequent cell growth inhibition[94,95]. Further to this, UV light is known to decrease the expression of pro-inflammatory cytokines, such as IL-1, IL-23[96]. Phototherapy has a high safety record and can be used in children and pregnant women. However, due to limited number of phototherapy centres and requirement for frequent treatments (typically 3 times weekly), phototherapy is often a very inconvenient option for patients.

Systemic agents, such as Methotrexate, Ciclosporin and Acitretin are used for the treatment of moderate to severe psoriasis. Although these drugs can be effective and have been shown to cause improvement, they pose certain risks. For example, Methotrexate is a ubiquitous psoriasis

treatment therapy and is also useful when arthritis is present. It interferes with folate biosynthesis and can inhibit T-cell and B-cell activity[97,98]. However, methotrexate use is associated with hepatotoxicity, nausea, diarrhoea and fatigue[99]. Ciclosporin on the other hand can lead to the development of skin malignancies and lymphoma, especially in those predisposed, e.g. due to previous UV therapy[100] and therefore, patients need to be monitored closely and made aware of the possible associated risks.

1.2.3 Biologics

Biological agents can target specific immune mediators, which participate in the immunopathology of psoriasis, for removal. Biological agents are complex, large molecular-weight proteins, most often monoclonal antibodies or receptor fusion proteins, which act extracellularly. Most are highly effective therapies for the treatment of medium to severe psoriasis, which have led to rising treatment expectations by patients. Further to this, this has now challenged the gold standard PASI75 paradigm (75% reduction in PASI score) to now consider PASI90 and PASI100 (90%/100% PASI reduction respectively) as more appropriate endpoints, where patients achieve near- or complete clearance[55a,101].

The development of biological agents has progressed with our understanding of the immunopathogenesis of psoriasis. The oldest biologics for the treatment of psoriasis, namely alefacept and efalizumab, would target molecules important for T-cell migration and activation (e.g. CD2, CD11a)[102,103].. New agents, which target pro-inflammatory cytokines, such as TNF, were developed and are now known as first generation biologics: etanercept, infliximab, and adalimumab, which remain in use today. TNF is key pro-inflammatory cytokine in the pathogenesis of many autoimmune conditions, including psoriasis. It promotes the expression of pro-inflammatory mediators, such as IL-1, IL-6, CXCL8 and adhesion molecules, such as P- and E-selectin. Etanercept is a TNF targeting fusion protein, which consist of two TNF receptor units and an Fc portion of human IgG. It was first approved for the treatment of psoriatic arthritis and later psoriasis. Trials showed that 47-49% of the patients, who received 50mg of Etanercept biweekly, achieved PASI75 by week 12, compared with placebo (3-4%)[105]. Reduction of epidermal thickness, T-cell skin infiltration and proliferation, markers of inflammation (e.g. cytokines) were also reported in those patients[105]. Infliximab is a chimeric IgG1 monoclonal antibody against TNF which has performed well in trials[106] and is often used

for more severe cases where PASI is above 10[20,55a]. Adalimumab is a fully human monoclonal IgG1 antibody that targets TNF. Infliximab and Adalimumab therapies lead to a significant reduction of psoriasis symptoms[107]. Although the three TNF inhibitors share the same target, their molecular structure and efficacy differs. Studies comparing the three TNF inhibitors have shown evidence in support of Infliximab being the most effective of the three in managing psoriasis pathogenesis; however, Infliximab is given as an intravenous infusion as is thus less practical in general than self-administered adalimumab[106,108]. Some of the frequent adverse effects caused by TNF inhibitors include injection site reactions, upper respiratory tract infections, abdominal pain.

Biological therapies targeting other cytokines have also been made available and are known as second generation biologics: Ustekinumab, secukinumab, ixekizumab, brodalumab, ustekinumab, guselkumab, risankizumab and tildrakizumab[55a]. They target pathways important for T-cell differentiation and function. The role of T-cells in psoriasis pathogenesis has been confirmed by multiple studies. The first such study showed that selective T-cell apoptosis leads to lesion resolution, without affecting keratinocyte survival[109]. Ustekinumab targets the shared protein subunit p40 of IL-12 and IL-23 and therefore inhibits Th17 pathways, and to a lesser degree Th1 pathways[110]. Studies showed that CD4+Th17 T-cells and associated cytokines were elevated in psoriasis and that intradermal injection of IL-23 stimulates psoriasiform plaque development in mice[111,112]. These findings were confirmed by later studies and led to the realisation that the pathology of psoriasis is predominantly driven by Th17 pathways, rather than Th1 pathways. This was evidenced by the generally higher efficacy of IL-17 inhibitors compared to first-generation biologics, [114]. Secukinumab is an anti-IL17A targeting fully human IgG1 monoclonal antibody[113]. When compared to earlier biologics, secukinumab has a fast onset of action, is highly effective and has higher PASI90 and PASI100 response rates than most anti-TNF therapies[115]. Further to this, phase III trials showed that 75.9–86.7% of the patients, who received 300mg of Secukinumab (once weekly for 4 weeks, and then once every 4 weeks), achieved PASI75 by week 12, compared with placebo (0-4.9%)[115,116]. Ixekizumab is a humanised IgG4 anti-IL-17A monoclonal antibody and it is additionally possible to target the action of multiple cytokines of the IL-17 family of cytokines at the same time, via brodalumab (fully human anti-IL-17RA IgG2 monoclonal antibody)[118]. It is believed that the success of IL-17 neutralising therapies is partially attributable to a reduction in neutrophil numbers in skin [119]. More recently, IL-23 specific anti-IL-23p19 neutralizing antibody therapies have been developed. Guselkumab and risankizumab are licensed for psoriasis and are humanised monoclonal antibodies targeting anti-IL-23p19 and have shown to have better efficacy than

ustekinumab, whilst the p19-IL23 inhibitor Tildrakizumab appears to have comparatively lower efficacy, though head-to-head trials are yet to be conducted[55a,87,88].

It is important to note that biologics rarely cause severe adverse effects in clinical practice; however, they can increase the risk of infections (sepsis; reactivation or enhancement of Mycobacterium tuberculosis), malignancies, and in the case of anti-TNF drugs congestive heart failure[120]. Although biologics are highly efficacious, some patients do not respond to a specific biologic (primary failure) or lose responsiveness over time (secondary failure), and the rates vary greatly depending on the biologic[121]. This is believed to be due to the generation of antibodies against the drug, or possibly the development of compensatory pathways. Additionally, they are generally very costly, which can limit access in some patient groups and in some geographical areas.

1.2.4 Small molecule inhibitors

Small molecule inhibitors represent a comparatively more recent group of treatments for the management of psoriasis. These inhibitors have a small molecular weight (<1kDa); can be administered topically or orally (whereas biologics are injected); and can modulate pro-inflammatory cytokine production. Small molecule inhibitors act intracellularly and block signalling pathways that lead to cytokine production[122]. Approved small molecule therapies for the treatment of psoriasis include phosphodiesterase 4 (PDE4) inhibitors (e.g. Apremilast), Janus kinase (JAK) inhibitors, sphingosine 1-phosphate (S1P) receptor agonists. Small molecules represent a useful alternative for patients who respond poorly to systemic or biologic therapies[123]. As the findings in this thesis concern the effects of Apremilast on leukocyte migration, the signalling pathway and adjacent molecules affected by Apremilast will be discussed in more detail.

1.2.5 Apremilast

Apremilast is an orally available small molecule inhibitor of PDE4 and modulates the expression of mediators important in the pathogenesis of psoriasis[124,125]. Apremilast decreases the production of a plethora of pro-inflammatory factors, such as TNF, IL-23, VEGF, and increases anti-inflammatory IL-10[124,125]. Consequently, Apremilast reduces the manifestation of clinical

symptoms, such as skin thickening by limiting the rate of keratinocyte proliferation and is licensed for both plaque psoriasis and psoriatic arthritis.[126].

Multiple anti-inflammatory therapies for the treatment of psoriasis target specific pro-inflammatory molecules, such as TNF or IL-23[83,85]. Pro-inflammatory mediators and their expression is, however, a part of a larger inflammatory signalling cascade, which is dependent on complex intracellular molecular interactions. For example, the Adenosine 3',5'-cyclic monophosphate (cAMP)-signalling pathway controls a network of pro-inflammatory and anti-inflammatory mediators and is known to affect resident epidermal and dermal leukocytes (Figure 1.3)[128-130]. Although the role of the cAMP-signalling pathway in the context of skin inflammation is poorly understood, there have been reports of its activity being altered in psoriasis[131]. Furthermore, the successful treatment of psoriasis, via the targeting of PDE4, which degrades cAMP, with Apremilast, demonstrates the importance of the cAMP-signalling pathway in cases of psoriasis[78]. Therefore, the inhibition of PDE4 can be used for the modulation of cAMP-dependent pro-inflammatory and anti-inflammatory signalling networks in psoriasis. This is an important therapeutic approach, as it focuses on restoring immunological homeostasis, rather than targeting a single particular pro-inflammatory mediator.

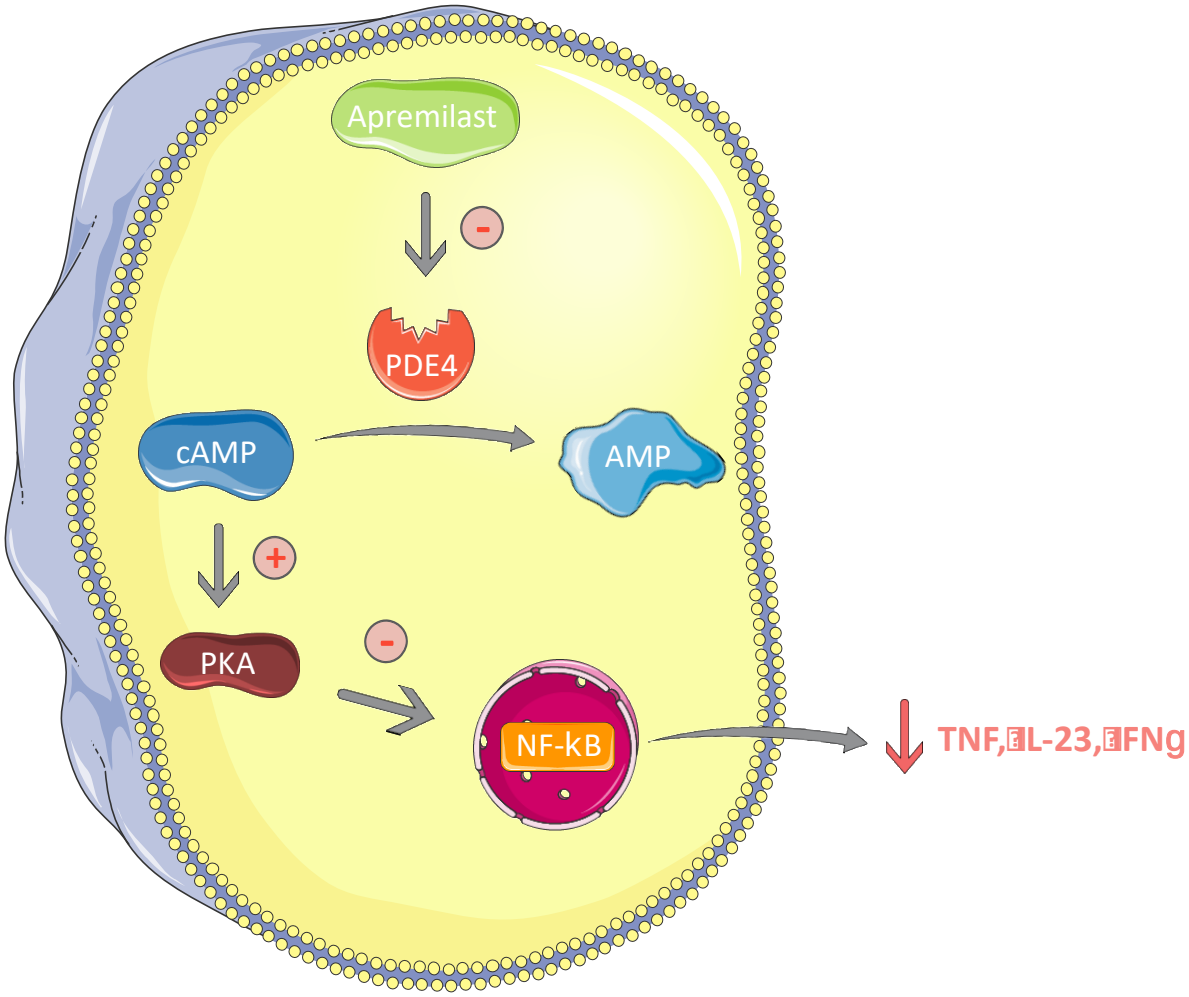


Figure 1.3 | The role of Apremilast in suppressing production of pro-inflammatory cytokines

Graphical representation of Apremilast-induced inhibition of PDE4-mediated degradation of cAMP. The resulting rising intracellular concentrations of cAMP lead to the activation of cAMP protein effector molecules; subsequent modulation of transcription factor activity (e.g. NF-κB) and pro-inflammatory cytokine production). Original figure, visual graphics taken from SmartServier.

1.2.5.1 The cAMP-signalling pathway

cAMP is a second messenger molecule important for the regulation of various cellular functions, such as metabolism, growth, differentiation, shape, gene and protein expression[132,133]. Second messenger molecules act to convert extracellular stimuli (first messengers), such as chemokines, hormones, neurotransmitters, received by cell surface receptors, to intracellular signals. cAMP is generated when a first messenger molecule binds to an adjacent G-protein-coupled receptor (GPCRs). This, in turn, leads to the stimulation of a downstream enzyme, known as adenylyl cyclase (AC)[137]. ACs located at the plasma membrane, downstream of GPCRs, are recognised as transmembrane ACs (tmACs) [136]. A class of ACs, known as non-canonical soluble ACs (sACs), are activated by adenosine triphosphate (ATP) and calcium and can be found within the cytoplasm (mitochondria, centriole, nucleus)[134,135]. ACs can catalyse the cyclisation of ATP to yield cAMP, alongside pyrophosphate[138]. cAMP can amplify and transduce signals by utilising cAMP-effector proteins, such as protein kinase A (PKA) and exchange proteins activated by cAMP (EPACs)[139]. Gene transcription and subsequent cellular reaction is evoked when, for example, PKA or EPACs phosphorylate a transcription factor, such as cAMP-response element-binding protein (CREB)[140].

The activity of cAMP-signalling is tightly regulated by the family of phosphodiesterase (PDE) enzymes[137]. PDEs hydrolyse cAMP into nucleoside monophosphate, rendering cAMP biologically inactive[141]. Importantly, recent studies have suggested that the intracellular localisation of PDEs can be spatially organised, allowing cAMP regulation at a subcellular level[143]. A-kinase-anchoring proteins (AKAPs) serve to localise PKAs and PDEs and can target PDEs to specific cellular microdomains, in close proximity to effector molecules, such as PKAs[143-145]. This can reduce microdomain cAMP levels and terminate a PKA-mediated signal. Conversely, microdomains with low PDE activity, whereby PKAs are anchored to ACs, will facilitate quick cAMP effector activation[142]. The spatial PDE organisation into microdomains generates discrete intracellular pools of cAMP and cAMP effectors, allowing for a cell type-specific cAMP-signalling cascade signature[146]. cAMP activity is therefore highly dependent on cell type and effector signalling molecules. For the purpose of this thesis, cAMP-signalling will be discussed in the context of leukocytes.

1.2.5.2 The cAMP-signalling pathway in immune effector function

cAMP-signalling plays a direct role in leukocyte function. Elevated intracellular cAMP levels lead to supressed pro-inflammatory, and increased anti-inflammatory, state (including associated mediators) of leukocytes[147-152]. cAMP-signalling induces supressed function via the modulation of mediator generation and processes, such as phagocytosis[153]. Cytokines supressed via cAMP-signalling, include TNF, IL-23, IL-27, IL-17, IFN-β, IFN-γ, IL-12 family of cytokines, CCL3 and IL-1β, leukotriene B4[148-152].

As outlined earlier, cAMP can signal through multiple different effector molecules to achieve gene transcription and a subsequent cellular response. The signalling events and cAPM-effector protein involvement in supressing immune function is, however, incompletely understood. Studies suggest that cAMP-effector proteins can have redundant, independent or contradicting effects within the same cell[154,155]. For example, both PKA and Exchange protein directly activated by cAMP (Epac)-1 have been shown to mediate inhibition of pro-inflammatory cytokine production by T-cells and macrophages[156,157]. In contrast, studies suggest that IL-10 production regulation by cAMP and its effector molecules differs across leukocytes[156]. Regulation of phagocytosis by cAMP is even less well understood. Studies suggest that depending on the cell type, PKA and Epac-1 can counter-regulate phagocytosis via the modulation of phagocytic receptor expression[158,159]. The expression of many of the aforementioned pro inflammatory cytokines is controlled by the master regulator of inflammation – Nuclear Factor-kappaB (NF-κB). On a transcriptional level, the anti-inflammatory properties of cAMP-signalling have been partially attributed to its ability to inhibit NF-κB function via PKA[160,161]. However, studies have shown that this is cell-type-dependent and cAMP-PKA signalling can also stimulate NF-κB function[160-162]. Furthermore, modulation of NF-κB functions by this pathway is not exclusive to PKA and may involve EPACs[163]. The subcellular microdomain organisation of cAMP activity which implies cell-type specific functions could potentially explain the conflicting effects of cAMP-signalling and utilisation of different effector molecules. It is, however, incompletely understood what cAMP microdomain(s) plays a role in the regulation of leukocyte functions in the inflammatory process. The duality of the cAMP-effector protein system is further discussed in chapter 1.5.7.1 in the context of leukocyte migration. Nevertheless, the anti-inflammatory effects of cAMP-signalling, have made cAMP-degrading factors, such as PDEs, promising therapeutic targets for the treatment of inflammatory conditions[78].

1.2.5.3 PDE4 inhibition for the treatment of psoriasis

As discussed, cAMP-signalling could be beneficial to the attenuation of pathological pro-inflammatory responses and therefore restore the pro-inflammatory/anti-inflammatory balance in psoriasis. PDE enzymes of different families exist and regulate the activity of cyclic nucleotides, such as cAMP, in different cell types and biological processes. PDE enzymes function in a tissue-specific manner thus adding another mechanism to fine tune this system[164,165]. In leukocytes (including T-cells and neutrophils) and keratinocytes, PDE enzymes of type-4, -7, and -8 have a significant role in specific cAMP degradation[166-168]. However, PDE4 has been shown to have the most prominent role in immune cell functions and chronic inflammation[125,169]. PDE4 enzymes have at least 35 isoforms, whose expression change with cell differentiation [170,171]. Our understanding of PDE4 isoform expression regulation among cell types and its effects on cell function, such as T-cell proliferation, has grown over the years. For example, it has been shown that the T-cell receptor can recruit PDE4A4 and PDE4D1. However, these PDE4 isotypes can exert different effects: PDE4D activity can halt T-cell proliferation, whereas PDE4A activity does not affect T-cell proliferation at all[172]. It is still unclear how PDE4 may impact specific leukocyte effector functions, such as cytokine production, migration, phagocytosis or apoptosis. Nevertheless, it is an attractive therapeutic target and for this purpose PDE4 inhibitors, such as Apremilast, have been developed[125]. No other PDE4 inhibitor has been more extensively researched than Apremilast[78,124,125]. Apremilast binds to PDE4, which leads to PDE4 inactivation and elevation of cAMP-mediated PKA activation[78]. Elevated CREB and inhibited NF-κB transcription factor activity has been noted following Apremilast treatment, which is in agreement with the signalling pathways discussed earlier[173,174]. The safety and efficacy of Apremilast have been evaluated by three placebo-controlled trials (ESTEEM1, ESTEEM2, and LIBERATE)[175-177]. Patients included in the trials were selected on the basis of criteria such as PASI score >/= 12 (moderate to severe psoriasis) and given 30mg Apremilast or placebo. In the ESTEEM1 trial, 33.1% of the Apremilast-treated group and 5.3% of the placebo-treated group achieved 75% reduction in PASI score (so called PASI75) at the study primary endpoint[175]. Similar results were demonstrated by the ESTEEM 2 and LIBERATE trials[176,177]. Importantly, the LIBERATE trials also compared the efficacy of Apremilast to the efficacy of etanercept[177]. No statistically significant differences were noted between the Apremilast and etanercept treatment groups. These groups achieved a significant improvement of their PASI and DLQI scores when compared to the placebo group[177]. This data suggest that Apremilast is efficacious in treating psoriasis and is comparable in effect to an older-generation biologic. Following these trials,

Apremilast was approved by the Food and Drug Administration (FDA) for the treatment of psoriasis in 2014[178]. Additionally, Apremilast is licenced for the treatment of psoriatic arthritis, and has an effect on neutrophilic disorders including Behçet's disease[178a]. However, the direct effect of Apremilast on leukocyte effector functions, such as migration, phagocytosis, cytokine production, and leukocyte skin infiltration remain poorly defined. Further to this, a clear molecular mechanism of action of Apremilast has not been defined yet. The better understanding of its mode of action could help understand why some patients respond very well to treatment, whilst some do not thereby enabling more rational targeting of those patients most likely to benefit, and enable the utilisation of Apremilast for the treatment of a potentially broader range of conditions in which Apremilast-targeting pathways play a key pathogenic role.

1.3 The genetic basis of psoriasis

Psoriasis aetiopathogenesis depends on complex genetic, environmental and immunological interactions. The complexity of these interactions is exemplified by the different clinical variants of psoriasis, highly variable disease prognosis among patients as well as the unpredictable manner in which some respond to a therapy whilst others do not. The current understanding, supported by a multitude of studies, is that a complex, multifactorial genetic component to psoriasis exists[179-182]. As is the case with many immune-driven conditions, the genetic background of psoriasis remains incompletely understood.

1.3.1 Genetic inheritance of psoriasis

Our understanding of psoriasis inheritance comes primarily from population-based, family-based, and twin studies[179-182]. It is important to note that a genetic investigation requires a clearly defined phenotype. Different clinical variants of psoriasis exist, therefore in order to decrease heterogeneity, most psoriasis genetic studies consider patients with chronic plaque psoriasis. Three large epidemiological population based-studies (Sweden, Germany, the Faroe Islands) first revealed that relatives of patients with psoriasis were at higher risk of developing psoriasis than the general population[183-185]. Furthermore, there have been reports of strong familial clustering of psoriasis. For example, family studies demonstrated that the lifetime risk of developing psoriasis increases by 7- or 16.25-fold if one or both parents have psoriasis,

respectively[186]. These findings are further supported by evidence of high concordance rate in monozygotic twins (35%) compared to dizygotic twins (12%)[187]. Although 100% concordance rate between twins is lacking, their onset of psoriasis (age, severity, plaque location) has been reported to be similar[187]. The mode of inheritance of psoriasis remains incompletely understood, but it is estimated that 60-90% of psoriasis susceptibility is attributed to genetic factors[180]. Although several reports have proposed an autosomal inheritance pattern, the studies discussed earlier suggest for a, now universally acknowledged, multilocus model of psoriasis[179,183-186]. Our understanding of diseased genetic loci that may confer risk of psoriasis has increased over the years; however, the lack of clear inheritance pattern and non-perfect concordance in monozygotic twins, supports the notion that psoriasis is a result of complex gene-environment interactions.

1.3.2 Genetic determinants of psoriasis

Linkage, association, and genome-wide association studies (GWAS) have been applied to map the psoriasis susceptibility (PSORS) loci in patients with psoriasis[188,189]. Of all PSORS loci identified, only PSORS1 has been robustly validated by multiple studies[189]. The PSORS1 locus is considered a major genetic determinant for psoriasis and spans the proximal Major Histocompatibility Complex (MHC) class I region on chromosome 6p21[190]. The PSORS1 region is dense in genes, such as HLA, important for the immune responses[191]. GWAS studies have allowed for this region to be finely mapped[191-193]. These studies identified HLA-C to be the most plausible causative gene, as it was highly polymorphic and showed consistent positive associations with psoriasis[191-193]. HLA-C encodes for an MHC class I molecule, important for the activation of CD8+ T-cell in the immune response, further highlighting possible associations with psoriasis. More specifically, HLA-Cw*0602 (encodes for HLA-Cw6) was identified as the susceptibility allele in the PSORS1 region and is associated with an early onset of disease[191,194]. These findings were consistent with older serological studies, which had also reported HLA-Cw6 associations with psoriasis susceptibility[192]. Although HLA-Cw6 frequency is higher in patients with psoriasis than healthy controls, it is still unknown how it might predispose to psoriasis[195]. Disease-associated alleles in the HLA-C expression regulation region have also been identified, suggesting for possible means to modify HLA-Cw6 expression in psoriasis[196]. One of several theories is that HLA-Cw6 may have a high binding affinity for psoriasis autoantigens. For example, studies have shown that the peptide binding grove of HLA-Cw6 has high affinity for LL-37[198]. Furthermore, there is evidence that HLA-Cw6 can present melanocyte autoantigens

(ADAMTS-like protein 5) to CD8+ T-cells[197]. However, the immunological implications of these findings in the context of psoriasis remain unexplored.

Genetic determinants, beyond HLA-C, that may confer psoriasis susceptibility have been identified within PSORS1 and other gene loci. For example, HLA-B is another MHC Class I molecule encoding gene, in the vicinity of PSORS1, that has shown positive psoriasis associations[199]. More specifically, the HLA-B*5701 allele has shown strong association signals[200]. The HLA gene region represents an attractive location for the identification of possible psoriasis causing genes, as demonstrated by multiple studies[196,198-200]. However, not all psoriasis risk-conferring genes are involved in antigen presentation. CDSN and CCHCR1 are highly polymorphic genes, within the PSORS1 spanning region, known to predispose to psoriasis[201,202]. While the function of CCHCR1 is unclear, CDSN encodes for corneodesmosin, a protein involved in skin desquamation, a process which is altered in psoriasis[201]. It is, however, possible that those genes have a less important, secondary, association with psoriasis and could be co-inherited with HLA-Cw6.

Another locus with potential linkage, albeit weaker than PSORS1, to psoriasis susceptibility is located on chromosome 17q25 and is known as PSORS2[203]. A possible PSORS2 site has been recently described – a CARD14 gain-of-function mutation[204]. The mutation is said to lead to an increased activity of NF-kB and a subsequent heightened production of pro-inflammatory mediators, such as CXCL8, CCL20, and IL-36[204-206]. Further to this, GWAS studies have identified more genetic associations between psoriasis susceptibility and NF-kB signalling[207]. It is important to mention that genes related to various immune functions, such as modulation of Th17 responses, IL-23- and TNF-signalling, and more, have been identified[208,209]. For example, GWAS studies have mapped three psoriasis genetic determinant to the IL-23 receptor complex (IL-12B, IL-23A and IL-23R)[208]. IL-23 promotes and maintains Th17 responses which are known to drive psoriasis immunopathogenesis. Furthermore, transcriptomic analysis studies have implicated IL-1 family genes, including IL-36, in leukocyte recruitment and disease propagation in pustular psoriasis[206,206a]. Although these studies highlight the genetic complexity of psoriasis and predisposition to psoriasis, a definitive genetic determinant is yet to be identified.

1.4 Environmental risk factors in psoriasis

Environmental factors appear to be associated with the induction of inflammation in genetically predisposed individuals[13]. This notion is further highlighted by genetic studies, which have shown that genetic factors do not confer 100% risk of developing psoriasis[203,206]. Known extrinsic environmental triggers of psoriasis are certain medications (e.g. imiquimod, lithium, beta-blockers)[210]; Koebner phenomenon-triggering traumatic interventions (e.g. tattoo, surgery and other forms of physical trauma) and infections (e.g. Streptococcal)[211,212,215]. The mechanisms underlying the induction of psoriasis by medications are largely unknown. However, imiquimod can activate TLR-7 and TLR-8 and induce the production of IL-23/IL-17, cytokines regarded as critical in psoriasis pathogenesis[210]. Further to this, lithium has known pro-inflammatory activities[213]. In some cases, the biological targeting of IL-17, IL-23 or TNF can lead to cytokine imbalances and subsequent psoriasis development, known as paradoxical reactions[214]. Streptococcal-associated psoriasis induction is thought to result from the molecular similarities between streptococcal M protein and keratin (molecular mimicry)[215]. This could lead to aberrant T-cell activation and skin targeting[212,215]. Although streptococcal M protein is considered a psoriasis trigger in some patients, a definitive autoantigen is not apparent[215]. A relationship between the psoriasis-associated HLA-Cw6 and molecular mimicry-induced T-cell activation has not been established. It has also been suggested that bad lifestyle habit, such as smoking and drinking, may contribute to psoriasis development[216,217]. Several studies have addressed the possibility of interactions between genetic and extrinsic environmental factors in psoriasis development[218,221]. Although correlation studies have shown that smoking increases the risk of psoriasis in HLA-Cw6 carriers, a mechanism underpinning such interactions is yet to be identified[219]. Obesity is an intrinsic factor, which has been strongly associated with the onset of psoriasis development. However, it has been challenging to identify if obesity predisposes to psoriasis development or if psoriasis leads obesity, e g. through reduced exercise[220]. Although no direct link between obesity and psoriasis induction has been found, it has been suggested that adipose tissue may contribute to the pro-inflammatory state in psoriasis[222]. Further to this, obesity has been associated with psoriasis-predisposing epigenetic modifications[220]. DNA methylation, histone modification and non-coding RNA are epigenetic modifications associated with psoriasis[221]. For example, it has been shown that DNA methylation can lead to the inhibited production of Secreted frizzled-related protein 4 (SFRP4) in psoriasis[223]. SFRP4 is a negative regulator of keratinocyte proliferation and pro-inflammatory cytokine production. Nevertheless, the environmental factor-driven mechanisms of psoriasis induction and their interactions with genetic factors is incompletely understood.

1.5 Immunology

1.5.1 Overview

The field of immunology has rapidly evolved over a course of over 50 years. The generation of immune memory against foreign pathogens, such as polio viruses, has revolutionised modern medicine[225]. Although the concept of immunology is only a comparatively recent medical discovery, there are records, which date back to 1549, referencing variolation in China[226]. These practices were later employed in the Ottoman empire (1718) and Europe, where Edward Jenner introduced the smallpox vaccine in 1798[227,228]. However, it was not until 100 years later when Louis Pasteur and Robert Koch came to the realisation that foreign pathogens ('germs') can cause disease, in the 'germ theory of disease'[229,230]. Since then the cellular and humoral constituents of the immune response have been defined and revised, allowing the development of modern vaccines and immune-modulating medicine.

The immune system provides the host with means to mount a reciprocal response against harmful opportunistic pathogens, toxic substances, intrinsic stress, neoplasms[231]. A complex recognition receptor network is in place to allow for the detection of potential harmful antigens. This is critical for the generation of an antigen-specific immune response, activation of appropriate immune system constituents, and distinguishing self from non-self. The downstream events in the immune cascade are strictly regulated at multiple points in time. If left unchecked, the responses mounted by the host immune system can cause pathology, such as in allergies and autoimmunity. The immune responses are often described as 'innate' and 'adaptive', with scientists devoting particular functions to each pillar. Although this approach has its benefits, it is becoming increasingly clear that this perception is somewhat limiting[231,232].

1.5.2 The Lymphatic system and lymphoid organs

The lymphatic system is a part of the immune system, which provides a scaffold and microenvironment for the development of the immune responses[233]. The lymphatic system is comprised of a vast network of lymphatic vessels, which carry a clear fluid called lymph.

Furthermore, specific lymphoid organs and tissues support the development ofleukocytes and facilitate the physical encounter and interactions of these cells[235]. The organs of the lymphatic system are conceived as primary (bone marrow, thymus), secondary (lymph nodes, spleen, mucosal-associated lymphoid tissues, Peyer's patches, adenoids, tonsils), and tertiary (inflammation-induced lymph node-like tissues). The bone marrow is a semi-solid, highly innervated and vascularised tissue in the cancellous bone compartment, where hematopoietic stem cells give rise to leukocytes. Importantly, B- and T-cell lymphocytes, the portion of leukocytes responsible for the generation of immune memory, originate from the bone marrow[236]. Whilst B-cell lymphocytes undergo development and maturation within the bone marrow, T-cells migrate to the thymus very early during development[237]. The thymus is a gland located above the heart and behind the sternum, where T-cells commit to their linage and undergo a rigorous process of negative and positive selection. The maturation of lymphocytes is a strictly regulated process which aims to eradicate self-reactive cells and therefore prevent autoimmunity[238]. Following maturation most leukocytes egress to the periphery and survey tissues for potentially harmful antigens; however, lymphocytes aggregate within secondary lymphoid organs. Secondary lymphoid organs are sites where immune responses are generated[239-241]. The structure of secondary lymphoid organs allows for the controlled activation of zone-compartmentalised lymphocytes by antigen-bearing leukocytes (antigen presenting cells)[239]. Lymph nodes are strategically positioned throughout the lymphatic network, at sites where pathogen entry is more likely to occur (e.g. neck, groin, abdomen). The lymph nodes are served by lymphatic vessels, which deliver antigens and antigen presenting cells via the lymph[242]. Furthermore, lymph nodes act as filter points for the lymph, which drains multiple organs and can therefore carry harmful antigens. The spleen serves similar functions but is served by the splenic artery, rather than lymphatic vessels. The spleen therefore filters blood, rather than lymph, and protects against systemic infections[243]. Mucosal-associated lymphoid tissues, such as gut- and skin-associated lymphoid tissues, are sites which provide local protection and maintain immune homeostasis (e.g. tolerance to food antigens and commensals)[240]. Tertiary lymphoid organs (TLOs) develop as a result of chronic conditions, such as certain microbial infections, autoimmune diseases, graft rejection. They resemble the cellular and molecular composition of secondary lymphoid organs[244]. Furthermore, TLOs develop B-cell and T-cell follicles, with chemokines governing compartmentalisation, antigen presentation and cell localisation. However, it remains unclear what the contribution of TLOs to pathological inflammatory responses is[244].

1.5.3 Pattern recognition receptors

Leukocytes perceive and produce a response to events in the microenvironment by utilising various receptors, including pattern recognition receptors (PRRs)[245]. PRRs are highly specialised proteins which recognise pathogen-associated molecular patterns (PAMPs) or molecules released by stressed or dying cells (danger-associated molecular patterns (DAMPs)). Leukocytes express a variety of PRRs, which allows them to elicit an appropriate response against a broad range of pathogens. The functional attenuation of PRRs leads to reduced pro-inflammatory responses and ability to clear pathogens[246,247].

Four main sub-families of PRRs are recognised - Toll-like receptors (TLRs); nucleotide-binding oligomerization domain (NOD)-leucin rich repeats (LRR)-containing receptors (NLRs); retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), and C-type lectin receptors (CLRs)[245]. However, for the purpose of this thesis, and because TLRs have been implicated in psoriasis pathogenesis, TLRs will be discussed in greater detail.

1.5.3.1 Toll-like receptors

TLRs were identified in drosophila fly genetic studies, whereby mutations in the Toll signalling pathway led to inability to clear fungal infections[247]. TLRs were one of the first PRR to be discovered, an event which has redefined our understanding of the immune system[248]. TLRs are transmembrane glycoprotein receptors with a horseshoe-like shaped ectodomain for ligand binding, composed of leucine rich repeats[249,251]. The transmembrane portion of TLRs is a single membrane spanning region with a length of approximately 20 amino acids. The cytoplasmic domain is known as the Toll/IL-1 receptor (TIR) domain and serves to initiate intracellular signalling[250,251]. TLRs are part of the IL-1 receptor/TLR superfamily, as they share their transmembrane region and a highly homologous cytoplasmic domain with IL-1 receptors[252]. The TLR family in humans is comprised of 10 members (TLR1-TLR10), which can be expressed on the cell surface or intracellularly (e.g. endosomes, lysosomes)[253,254]. Importantly, the localisation of TLRs is related to their function. TLR members that recognise lipids or proteins are generally expressed on the cell surface (TLR1, TLR2, TLR4-6, TLR10), whereas intracellular TLR (TLR3, TLR7-9) recognise nucleic acid motifs[253,254]. TLRs can also exist in a soluble form and reduce or enhance the reactivity of their membrane-bound form[255]. Different TLRs have evolved to

recognised different pathogen motifs. For example, TLR4 recognizes bacterial lipopolysaccharide (LPS); TLR5 recognizes bacterial flagellin; TLR2, TLR1, TLR6 recognize a wide variety of PAMPs including lipoproteins, peptidoglycans, zymosan, mannan[247,254,256]. TLR3 recognizes self and viral double-stranded RNA, whereas TLR7 recognizes single-stranded from viruses[257]. TLR activation results in the recruitment of TIR-containing intracellular signalling adaptor molecules, such as myeloid differentiation primary response gene 88 (MYD88), TIR domain containing adapter inducing interferon-β (TRIF), TIR domain containing adaptor protein (TIRAP)[258].

Upon activation, TLRs induce downstream signalling by recruiting several TIR-containing intracellular adaptor molecules, such as myeloid differentiation primary response gene 88 (MYD88), TIR domain containing adaptor protein (TIRAP), TIR domain containing adapter inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM) [258-262]. These adaptor molecules participate in TLR-induced intracellular signalling by utilising two distinct pathways, which are either MyD88- or TRIF-dependent[258,262]. TIRAP and TRAM act as signal mediators by recruiting MyD88 or TRIF adaptor molecules, respectively, to cell surface TLRs[259-261]. The intracellular cascade results in the upregulation of pro-inflammatory factors (e.g. IL-1, IFN, IL-6, TNF), via NF-κB (MyD88) or NF-κB and IRF3 (TRIF) activation[262,263]. All TLRs but TLR3 signal through MyD88. TLR3 signals trough TRIF; TLR4 can signal through both MyD88 and TRIF[258,260]. It has been demonstrated the TLR4 expression is highly elevated in psoriasis epidermis[264].

1.5.3.2 Other pattern recognition receptors

NOD-like receptors (NLRs) are another class of PRRs, expressed by epithelial and immune cells[265]. NLRs respond to microbial and danger signals in the cytosol[266,267]. NLRs consist of a NOD domain, N-terminal interaction domain, and C-terminus leucine-rich repeats. NOD1 and NOD2 are NLRs, which activate NF-kB and MAPK signalling pathways[268]. Another important NLR is NALP3, which is responsible for the inflammasome activation and subsequent IL-1β and IL-18 production[269]. The inflammasome shall not be further discussed as it is not the focus of this thesis.

C-type lectin receptors (CLRs) are transmembrane PRRs, expressed by leukocytes, such as monocytes, macrophages, and dendritic cells. CLRs respond to carbohydrates, including mannose

and fucose, on pathogens, such as bacteria, fungi, and viruses[270]. NLRs induce pathogen internalisation, degradation and antigen presentation and are therefore important for both innate and adaptive immune responses[271].

RIG-like receptors (RLRs) are intracellular cytoplasmic PRRs, expressed by leukocytes. RLRs recognise viral double stranded RNAs, associated with viral replication[272]. All RLRs consist of an RNA helicase core, for dsRNA recognition, and a zinc-binding C-terminal domain. Most RLRs, also have an N-terminus CARD domain to initiate a signalling cascade[273]. There are three main RLRs, including RIG-I, melanoma differentiation factor-5 (MDA5), and laboratory of genetics and physiology-2 (LGP-2). RLR activation leads to the induction of 1) pro-inflammatory cytokines, most notably type I IFNs, to limit viral replication and 2) antigen-specific adaptive immune responses[274].

1.5.4 Cytokines

Cytokines are low molecular weight, non-structural polypeptides/peptidoglycans produced for the purpose of intercellular communication. Cytokines coordinate and integrate functions of multiple cell types in the context of homeostasis and inflammation by ligating specific cell surface receptors. Cytokines are regulators of various aspects of the immune response, such as cell production, activation, differentiation, proliferation, migration[275].. On the basis of structural and functional properties, cytokines can be categorised into different groups[276]. This subchapter shall give a brief overview of different cytokine families and their function in immune responses. An in-depth discussion of the chemotactic family of cytokines will be given, as chemokines are strongly featured in this thesis.

1.5.4.1 Interferon family of cytokines

Interferons (IFN) can be divided into three major groups – type I, type II, and type III. Type I interferons include IFNα and IFNβ and are important for the protection against viral insults and neoplasms[277,278]. Type I IFNs are produced rapidly upon microbial challenge by TLR-6, -7, -9 and-10-expressing cells, such as macrophages, fibroblasts, plasmacytoid dendritic cells (pDCs)[279-281]. Type I IFNs can enhance the lytic potential of natural killer cells, enhance MHC I

expression on infected cells, and inhibit viral replication[282-284]. Type II IFN is known as IFNγ andis important for macrophage activation. IFNγ and is secreted by natural killer cells and T-cells, under the influence of IL-12 and IL-18[286]. Macrophage activation can in turn stimulate T-cell responses, aiding pathogen eradication. IFNγ is also important for the induction of Th1 responses and stimulation of apoptosis of infected cells and MHC expression[287,288]. Type III IFNs, such as IL-28/29, are important regulators of anti-viral immune responses[289]. The dysregulated production of IFNs could therefore be detrimental in autoimmune conditions.

1.5.4.2 Tumour necrosis factor family of cytokines

The tumour necrosis factor (TNF) superfamily and TNF receptor superfamily include over 40 effector molecules, which participate in a variety of inflammatory reactions[290]. The prototypical member of this family, TNFα (also known as TNF), is produced by a multitude of cells, such as macrophage, monocytes, T-cells, Natural killer (NK) cells, keratinocytes[291]. TNF production can be induced via TLR ligation by PAMPs or as a result of cytokine signalling (e.g. IL-17)[292,294]. TNF receptors (TNFR) can be soluble or membrane bound and binds to TNFR1 (expressed by leukocytes and stromal cells) and TNFR2 (expressed by T- and B-cells)[293]. TNF acts to increase adhesion molecule expression, cytokine and chemokine production, induce apoptosis, and increase leukocyte microbicidal functions[295]. TNF has been implicated in various autoimmune conditions[296,297]. As a consequence, multiple, successful TNFα targeting therapies have been developed for the treatment of such diseases and remain widely used[81-84].

1.5.4.3 Interleukin-1 family of cytokines

The interleukin (IL)-1 family of cytokines includes IL-1a, IL-1b, IL-18, IL-36, IL-37, and IL-1 receptor antagonists (IL-1Ra, IL-36Ra, IL-38)[298]. Of these cytokines, IL-1β is a potent activator of inflammatory responses and is produced by cells, such as macrophages, monocytes, neutrophils[299-301]. IL-1β expression is usually induced by the ligation of PRRs, such as TLRs, by bacterial and viral molecules[300]. However, IL-1β along with most of the IL-1 family members, is synthesised as a pre-cursor which needs to be cleaved in order to become activated[302]. Cleavage of pro-IL-1β is carried out by caspase-1, which is a part of the inflammasome protein

complex, and can be activated via PRR engagement[302,303]. IL-1α can function as an active cytokine in its precursor form (i.e. does not need cleaved)[304]. Both cytokines signal through the same receptors, IL-1R1 and IL-1R2, and can trigger the MyD88-dependent pathway of NF-κB activation[305]. IL-1β can promote Th17 responses, activate neutrophils and macrophages, and induce pro-inflammatory cytokine production[299-301,306]. IL-1b has been heavily associated with autoimmune and autoinflammatory conditions[307,308].

1.5.4.4 Interleukin-2 family of cytokines

The IL-2 family of cytokines includes IL-2, -4, -7, -9, -15, and -21, which all bind to the common γ-chain receptor[309]. The IL-2 family of cytokines act as growth factors and aid the activation of differentiation of leukocyte progenitors and mature leukocytes. IL-2 is produced by CD4+ and CD8+ T-cells, B-cells, DCs, natural killer cells[310,311] and is important for the development of Th1, Th2, Treg responses but has been shown to inhibit Th17 differentiation[311,312]. It also stimulates T-cell proliferation and enhances its own receptor expression, with the latter being a quality of multiple IL-2 family cytokines. IL-4 has been implicated in immune responses against extracellular parasites[313]. IL-4 can stimulate Th2 responses and induce B-cell class-switching to IgE[314]. IL-7 is a homeostatic cytokine, critical to the development of naïve T- and B-cells but also the proliferation and survival of mature T-cells[315]. The remainder of the cytokines from this family have been shown to be important for the activation and stimulation of T-cell (IL-9, IL-15, IL-21), NK (IL-15, IL-21), and B-cell (IL-21) responses[316-318].

1.5.4.5 Interleukin-12 family of cytokines

The IL-12 family of cytokines includes IL-12, IL-23, IL-27, and IL-35, which are all heterodimeric molecules[319]. Whilst IL-12 and IL-23 are pro-inflammatory mediators, IL-27 and IL-35 have been shown to be immune regulators[320,321]. IL-12 and IL-23 share a common p40 subunit and are both important for T-cell responses[320]. IL-12 is primarily produced by DCs, macrophages and B-cells and induces Th1 responses[322]. Th1 cell-derived IFNγ can further induce IL-12 production by APC, creating a positive feedback loop[323]. IL-23 is produced by DCs and macrophages and is important for the maintenance of Th17 populations and can induce its own receptor's expression[324]. Conversely, and in addition to IL-35, IL-27 has been shown to inhibit

Th17 development and drive IL-10 production[325,327]; however, IL-27 has been implicated in T follicular help cell (Tfh) responses[326]. IL-35 is regulatory T-cell (Treg)- derived and suppresses T-cell proliferation[327].

1.5.5 Chemokines

1.5.5.1 Overview

Chemokines are chemotactic cytokines which regulate leukocyte positioning and migration. Chemokines guide cellular migration in diverse scenarios, including leukocyte development, induction of cellular and humoral immune responses, leukocyte recruitment to the site of inflammation. Therefore, the immune system is critically dependent on chemokine function[328].

Chemokines and their receptors evolved at least 700 million years ago, evidence suggesting they first appeared in the genome of the jawless fish sea lamprey. The chemokine receptor repertoire then expanded in jawed vertebrae[328a]. Importantly, the functional interactions between chemokine receptors and their ligands have co-evolved over time. This has allowed for changes in one partner to be completed by ensuing changes in the other. An example of this is the CXCL12/CXCR4 pair, whereby their functional interactions have remained conserved throughout different vertebrae lineages. For example, the migration of primordial germ cells in zebra fish has been shown to be dependent on CXCL12/CXCR4 interactions[328b].

The chemokine family of cytokines is comprised of approximately 50 endogenous small molecule (8-12 kDa) chemokine ligands (Table 1.2). Chemokines are defined by the arrangement of their first N-terminus cysteine residues, which maintain the chemokine structure, via the formation of disulphide bridges[329-331]. On the basis of cysteine configuration variation, four chemokine subfamilies are recognised, which include CC chemokines, CXC chemokine, CX_3C chemokines, and XC chemokines. The cysteine residues in CC chemokines are juxtaposed, whereas in CXC chemokines they have a random amino acid (AA) located between them[329-331]. CX3C chemokines have three variable AAs between their cysteine residues, whilst XC chemokines have a missing first and third cysteines from their motifs. The chemokine nomenclature was revised in 2000 and now consists of subfamily designation (e.g. CC), letter L (for ligand), followed by a number (order of gene isolation)[329-331]. On the basis of their function, chemokines can be either

homeostatic or inflammatory[332]. Homeostatic chemokines are responsible for the maintenance of processes, such as lymphopoiesis and homing to secondary lymphoid organs. For example, T-cell development in the thymus is dependent on CCL21, CCL25, and CXCL12, whereas lymph node homing relies on CCL19/CCL21[333-335]. Inflammatory cytokines orchestrate leukocyte recruitment during acute and chronic inflammation. For example, CCL20 and CXCL8 induce T-cell and neutrophil trafficking, respectively, and are highly expressed during inflammation[336,337].

Table 1.2| A table of chemokines and their adjacent receptors.

Chemokine	Receptor	Function	Reference
CXCL1	CXCR2	Neutrophil trafficking	854, 855
CXCL2	CXCR2	Neutrophil trafficking	854
CXCL3	CXCR2	Neutrophil trafficking	856
CXCL4	CCR1	Monocyte trafficking	857
CXCL5	CXCR2	Neutrophil trafficking	858
CXCL6	CXCR1, CXCR2	Neutrophil trafficking	859
CXCL7	CXCR2	Neutrophil trafficking	860
CXCL8	CXCR1, CXCR2	Neutrophils	861, 855
CXCL9	CXCR3	T cell (Th1) trafficking	862, 863
CXCL10	CXCR3	T cell (Th1), eosinophil, monocyte, and NK cell trafficking	854, 863, 864
CXCL11	CXCR3	T cells (Th1) trafficking	863
CXCL12	CXCR4	Lymphocyte trafficking, bone marrow homing, blood vessel formation	865

CXCL13	CXCR5	B cell migration to splenic follicles	866
CXCL14	CXCR4	Natural CXCL12 inhibitor; also drives tissue-resident macrophage, natural killer cell, and dendritic cell migration	867
CXCL15	Unknown	Presumed to be active in mice only and act to recruit neutrophils	868
CXCL16	CXCR6	Drives neutrophil, Th1-cell, CD8+ T-cell, and Natural-killer T-cell migration	869
CXCL17	Undefined	Migration of dendritic cells and monocytes	869a
CCL1	CCR8	Migration of Th2 cells, macrophages, monocytes, regulatory T-cells	870
CCL2	CCR2	Monocytes, neutrophil migration	871
CCL3	CCR1, CCR5	Macrophage, Natural killer cells, T-cell migration	872
CCL4	CCR5	Macrophage, Natural killer cells, T-cell migration	873
CCL5	CCR5	Macrophage, Natural killer cells, T-cell migration	874
CCL6	unknown	Unknown; identified in mice only	875
CCL7	CCR1-3	Monocyte recruitment	876
CCL8	CCR1-3, CCR5	Attracts monocytes, lymphocytes, basophils, and eosinophils.	877
CCL9/CCL10	CCR1?	Attracts Dendritic cells in mice	878

CCL11	CCR2, high selectivity for CCR3	Attracts eosinophils	879
CCL12 (mice only)	CCR2	Attracts eosinophils, monocytes, and lymphocytes	880
CCL13	CCR1-3, CCR5	Attracts monocytes, lymphocytes, basophils and eosinophils	881
CCL14	CCR1, CCR3, CCR5	Promotes chemotaxis of monocytes, eosinophils, and T lymphoblasts	882
CCL15	CCR1	Recruits monocytes	883
CCL16	CCR1, CCR2, CCR5, CCR8	Potentially involved in mast cell and eosinophil recruitment	884, 885
CCL17	CCR4	T cells (Th2 and regulatory T-cells)	864
CCL18	CCR8	CLA+ T cells and Th2 cells	886
CCL19	CCR7	Lymph node homing	365
CCL20	CCR6	T-cells (Th17)	337
CCL21	CCR7	Lymph node homing	237
CCL22	CCR4	Regulatory T-cells, Th2 cells	864
CCL23	CCR1	Endothelial cell migration; resting T-cells and monocytes	887
CCL24	CCR3	Recruits eosinophils	888
CCL25	CCR9	Thymocyte chemoattractant important for T-cell development	889

CCL26	CCR3	Eosinophil and basophil migration	890
CCL27	CCR10	T-cell skin homing	891
CCL28	CCR3, CCR10	T- and B-cell homing to mucosal tissues; eosinophil migration	892
CX3CL1	CX3CR1	T cells, monocytes, microglial, Natural-killer cell migration	893
XCL1 and XCL2	XCR1	Dendritic cell migration; importance in cross-presentation	894; 895
ACKR1	CCL2, 5, 7, 11, 13, 14, 17; CXCL1, 2, 3, 5, 8, 11	Transport of chemokines across endothelial cells; regulates chemokine abundance in blood; controls neutrophil abundance in blood	339, 342, 364, 364a, 853
ACKR2	CCL2, 3, 3L1, 4, 5, 7, 8, 11, 12, 13, 17, 22	Chemokine scavenging and internalisation	
ACKR3	CXCL11, 12	CXCL12 scavenging; regulation of CXCR4 expression and signalling	
ACKR4	CCL19, 21, 25; CXCL13	Regulation of dendritic cell migration via CCL19/CCL21 scavenging	

Original table.

Chemokine action is mediated by a family of rhodopsin-like 7-transmembrane G-protein–coupled receptors (GPCRs). The chemokine receptor (CKR) family encompasses 20 members divided into four subfamilies on the basis of their chemokine subfamily specificity[339]. There are 10 CCRs, 8 CXCRs, 1 CX_3CR and 1 XCR, which are expressed on a plethora of cells and have a high cytokine binding and response variability. Similarly, to their ligands, CKRs are important during inflammatory processes and for the maintenance of homeostasis[339]. CKRs specificity is complex, as most of the chemokine ligands can bind multiple receptors and chemokine receptors can bind different ligands, creating a highly promiscuous system[338]. This characteristic is prominent in inflammatory chemokine/CKR interactions and has likely evolved to counter pathogen subversion mechanisms of the host chemokine system[338]. Further to this, high promiscuity allows for a fine-tuning of the chemokine system and its role in leukocyte migration. Only a minority of chemokine/CKR pairs are involved in monogamous interactions (e.g. CCL20/CCR6)[340]. Although, multiple ligands can bind a given receptor, chemokine receptor affinity may vary, resulting in biased signalling and functional selectivity[341]. In addition to the conventional CKRs, atypical CKRs (ACKRs) have also been recognised[342]. These receptors lack the conventional CKRs canonical DRYLAIV motif within the second intracellular loop and fail to induce classical CKR-dependent cellular responses. ACKRs function to target chemokines to intracellular lysosomes for degradation, thus limiting cell exposure to chemokines and subsequent inflammation. ACKRs can also transport chemokines across biological barriers. These features allow ACKRs to regulate chemokine/CKR interactions.

1.5.5.2 Chemokine receptor signalling

Each leukocyte expresses various chemokine receptors, which serve to integrate extracellular signals and direct subsequent cellular function[339]. Most notably, chemokine receptors activate leukocyte migration by engaging intracellular signalling molecules in a multi-step process[339]. The classical view of chemoattractant-induced cell migration involves the activation of a G-protein signalling pathway. Ligation of a GPCR triggers conformational changes in the 7-transmembrane region and the subsequent signalling cascade[328,339]. The conformational changes facilitate the binding of intracellular heterotrimeric $G_{\alpha\beta\gamma}$ protein to the receptor loop region. The activated GPCR can then induce guanosine diphosphate (GDP) to guanosine triphosphate (GTP) exchange at the nucleotide binding site of the $G\alpha$ subunit[340]. The $G\alpha$ subunit proteins have been broadly divided into four subfamilies - $G\alpha s$, $G\alpha i/o$, $G\alpha q/11$, and $G\alpha 12/13$. Chemokine receptors predominantly associate with $G\alpha i$ proteins; however,

interactions with Gαq proteins have also been noted[344,345]. The GDP to GTP exchange leads to the disassociation of the Gαβγ trimer into the Gβγ dimer and GTP-bound Gα subunit. The disassociated subunits can modulate the production of second messenger molecules and trigger separate downstream signalling pathways. For example, the Gα subunit has been associated with the activation of calcium channels and the inhibition of cAMP[346]. The induction of leukocyte migration has, however, been attributed to the propagation of signalling pathways by the Gβγ heterodimer. Gβγ can trigger phospholipase C-β activation and subsequent cytosolic elevation of inositol trisphosphate (IP3) and diacylglycerol (DAG)[348,350]. In turn, IP3 mobilises Ca^{2+} from intracellular stores, which in conjugation with DAG, induces activation of various protein kinase C (PKC) isoforms[348,349]. These molecules participate in cell migration by stimulating processes, such as actin polymerisation and GPCR regulation. The Gβγ heterodimer can also induce migration via the activation of phosphoinositide 3-kinase (PI3K)[347]. This is an important pathway for leukocyte migration, as PI3K inhibition in T-cells compromises cell migration[351]. PI3K activity results in the generation of second messenger molecules, such as phosphatidylinositol (4,5)-trisphosphate (PIP3), for the activation of downstream effectors (e.g. Rho family kinases)[354,355]. Evidence suggests that the Rho GTPase Rac is required for the PI3K-induced reorganisation of cytoskeleton and membrane ruffling during leukocyte migration[352]. Further to this, the leukocytes of $Rac2^{-/-}$ mice have been shown to be less able to migrate *in vitro*[353]. Importantly, other Rho GTPases, such as Cdc42, also participate in PI3K-induced leukocyte migration, suggesting for the existence of plasticity in the system[356]. This data show that PI3K and subsequent effector molecule activation is important for leukocyte migration. It is still not clear what the relative contribution of different PI3K isoforms to leukocyte migration is. PI3Kγ has been shown to be important, as PI3Kγ deficient mice fail to produce PIP3 and their neutrophils have an impaired response to CXCL8 (50-70% reduction). However, $PI3K\gamma^{-/-}$ mouse leukocytes do not fail to undergo actin polymerisation, following CKR ligation, and do not show complete failure to migrate[357]. Such findings demonstrate that other PI3K isoforms might become activated and modulate downstream signals important for leukocyte migration[358]. Other chemokine-receptor signalling pathways, downstream of PI3K, may result in the activation of kinases, such as Akt and mitogen-activated proteins kinases (MAPK)[359-361]. Nevertheless, the actin polymerisation and cellular morphological changes driven by these proteins, at the leading edge of the cell, allow movement. Furthermore, chemokine receptor-driven migration can be achieved via downstream signalling cascades, which may involve various second messenger and effector molecules. It is still to be elucidated what the relative contribution of these pathways to migration

is; what are the exact molecular participants in these pathways, and how interchangeable those molecules can be.

1.5.5.3 Regulation and modulation of chemokine receptor signalling

Chemokine receptors are expressed by various leukocytes, such as T-cells, DCs, and neutrophils. The chemokine expression profile of cells can differ, which usually reflects their particular function and response to different pathogens[328,332,334,339]. For example, Th1 cells are characterised by CXCR3, CCR5 expression, whereas Th17 cells express high levels of CCR6[340,362]. As discussed earlier, the expression of chemokine receptors is dynamic and a subject of modulation by environmental stimuli, allowing cells to elicit appropriate responses. For example, induction of CCR7 expression allows DCs to home to draining lymph nodes and induce T-cell activation[363]. Conversely, the regulation of chemokine availability, via ACKR-dependent scavenging, has been shown to be important for the resolution of inflammtion[364]. Therefore, chemokine receptor expression regulation is an important process in leukocyte migration. Regulation of chemokine receptor expression is achieved via receptor phosphorylation, desensitisation, and internalisation[365-367]. These processes are governed and triggered by molecules, such as β-arrestins and G-protein-coupled receptor kinases (GRKs)[366,368]. For example, when a chemokine is present at a high concentration it may cause a loss of CKR response, in a process known as homologous desensitisation. During homologous desensitisation, a member of the GRK family rapidly phosphorylates the cytoplasmic tails of CKRs, upon ligand binding[365,366,369]. This causes uncoupling of the G-protein from the receptor, blocking further receptor activation, and increases the affinity of CKR to bind β-arrestin proteins. β-arrestin proteins act as adapters at GPCRs, which target the receptors for internalisation via clathrin coated pits[370]. Receptors can be targeted for degradation or can recycle to the cell surface, depending on the affinity of β-arrestin/CKR interaction (e.g. high affinity interactions favour receptor degradation)[365]. The importance of this system has been highlighted by gene knock out studies. For example, GRK deficient neutrophils and mice show increased chemokine responsiveness and likelihood of developing experimental autoimmune encephalomyelitis, respectively[371,372]. Similarly, β-arrestin deficient neutrophils show reduced CXCR2 internalisation and increased migration rates in response to CXCL1[370]. Importantly, β-arrestins do not only participate in receptor desensitisation but can also trigger a second round of CKR signalling. β-arrestins can activate the MAPK cascade, which has been shown to increase

responsiveness of HeLa cells to CXCL12, *ex vivo*[370]. Further to this, β-arrestins can activate other pathways, such as the extracellular signal-regulated kinase pathway. Conversely to homologous desensitisation, the stimulation of a particular CKR via its ligands may lead to the desensitisation of multiple receptors, in process known as heterologous desensitisation[367]. This cross-desensitisation is explained by the rapid CKR phosphorylation, by kinases, such as PKA and PKC, and its subsequent uncoupling from G-proteins[367]. Therefore, the chemokine induced signal and cellular response are dependent on the interactions of multiple receptors, co-receptors, second messenger molecules, and adaptor proteins. The signalling molecules and pathways of the CKR system provide the cells with the means to rapidly respond to changes in the microenvironment in a ligand-specific manner. For example, different chemokines can induce distinctive cellular responses when signalling through the same CKR, by favouring one intracellular signalling pathway over another[373,374]. This is a phenomenon known as biased signalling, whereby one chemokine may signal through G-proteins, and another may preferentially activate β-arrestins[377]. Further to this, ligands can discriminate and engage different G-proteins and therefore fine tune the downstream signalling events. For example, CCL19 and CCL21 both induce G-protein binding upon CCR7 ligation and are important for leukocyte homing to the lymph nodes[375]. However, CCL19 induces 1) leukocyte migration at lower than CCL21 concentrations and 2) β-arrestin mediated CCR7 internalisation. Conversely, CCL21 signalling does not result in β-arrestin mediated receptor desensitisation, it rather utilises β-arrestins to activate the ERK1/2 phosphorylation[375,376]. It has been suggested that structural differences between the two chemokines cause them to utilise different GRKs during signalling, accounting for the distinct outcomes[378]. Importantly, biased signalling depends on CKR and signalling molecules availability and expression, which may be influenced by tissues, pathogens or other stimuli.

Chemokine receptors can form homo- and heterodimers, which provides another mode of CKR regulation. In this instance, CKR dimers establish a cross talk, which allows chemokines to alter the signalling response initiated by their non-respective receptor[379,380]. The cooperativity could be positive, whereby one part of the dimer enhances the responsiveness of the other to its respective ligand[380]. More often, however, the cooperativity is negative and ligand binding to one receptor inhibits agonist binding to the other[379]. It is thought that the co-operation, either negative or positive, is achieved by allosteric conformation changes in the CKR induced upon ligand binding.

1.5.5.4 Regulation of chemokine availability

As discussed earlier, the regulation of chemokine function is essential for effective clearance of pathogens and subsequent, timely resolution of inflammation. Chemokine function is regulated at many levels, including chemokine receptor expression and chemokine ability to bind to glycosaminoglycans (GAGs). GAGs are linear heteropolysaccharides, consisting of a repeating disaccharide unit, which can be attached to a protein and form proteoglycans[382]. They are expressed on endothelial cell (EC) surfaces and in the extracellular matrix[381]. Further to this, GAGs are differentially expressed on cells and tissues and their production enhances during times of inflammation[383]. Importantly, GAGs can bind chemokines, therefore causing chemokine immobilisation, retention and accumulation[384]. The GAG-induced localisation of chemokines, near the site of injury and chemokine production supports leukocyte recruitment[385]. This notion was supported by studies which identified heparan sulfate (a GAG) as an EC component[386]. Furthermore, CXCL8 was found to interact with EC surface projections and to have a GAG binding domain, which proved important for neutrophil migration[387]. GAG-induced chemokine immobilisation plays an important role during a process known as transendothelial migration (TEM)[385]. An important step in TEM is 'leukocyte arrest', whereby migrating leukocytes become activated. This step is critically dependent on leukocytes binding to GAG-immobilised chemokines on the endothelial surface[388]. Chemokines which participate in directing leukocyte migration to the site of inflammation can be produced by the blood vessel wall and underlying tissues[385]. Although chemokine-GAG interactions are poorly understood, it has been established that GAGs do not show the same binding affinity towards all chemokines[389,390]. It has been suggested that this is dependent on GAG length and degree of sulfation; however, it is likely that this concept is too simplistic to account for the complex geometries of chemokine ligands and their abilities to engage with GAGs[389]. Evidence suggests that chemokines oligomerise on GAGs and that GAGs further stabilise such structures[392]. This mechanism of mutual reinforcement could promote the accumulation of pro-inflammatory chemokines during inflammation and recruit more leukocytes[391]. *In vivo* studies demonstrated that GAG binding and subsequent oligomerisation are important for the activity of chemokines, such as CCL2, CCL4, and CCL5. Further to this, chemokine dimers and oligomers bind to GAGs with higher affinity than monomers and certain chemokines (e.g. CCL5, CCL21) have higher GAG-binding affinities than others (CXCL8)[390]. These mechanisms allow for the regulation of bound/unbound chemokine ratio and subsequent modulation of leukocyte migration, recruitment, and inflammation. The importance of chemokine availability in the propagation of diseases, such as psoriasis has been previously addressed. For example, scavenging and reduction of chemokine availability has been

associated with a reduction of inflammation in psoriasis[853]. However, the current understanding of chemokine spatial organisation, timing and regulation in inflammatory conditions remains limited.

1.5.6 Leukocytes

Leukocytes are the principal effector cells of the immune response. They are derived from a common hematopoietic stem cell (HSC) progenitor in the bone marrow (BM), during a process known as haematopoiesis. BM HSCs have a high self-renewal and differential potential and can give rise to all blood cell lineages[393]. However, this differentiation potential is lost as the HSC commits to a particular lineage in a step by step process. In leukocyte generation, HSC can commit to either a common myeloid or lymphoid progenitor fate[394]. The common myeloid progenitor will give rise to granulocytes (neutrophils, basophils, eosinophils), monocytes, macrophages, mast cells, myeloid-derived dendritic cells (mDCs). On the other hand, the common lymphoid progenitor can commit to a T-, B- or Natural killer-cell fate. These cells act in a well-coordinated and regulated sequential manner to provide optimal host protection. However, leukocytes (e.g. neutrophils, DCs, and T-cells) are abundantly present and drive pathogenesis in inflammatory conditions, such as psoriasis[15]. The subsequent section will give an overview of the basic biology of leukocytes involved in psoriasis pathogenesis (Figure 1.4 and 1.5) and related to the research described in this thesis. Their role in the context of psoriasis immunopathogenesis is discussed in chapter 1.5.9.

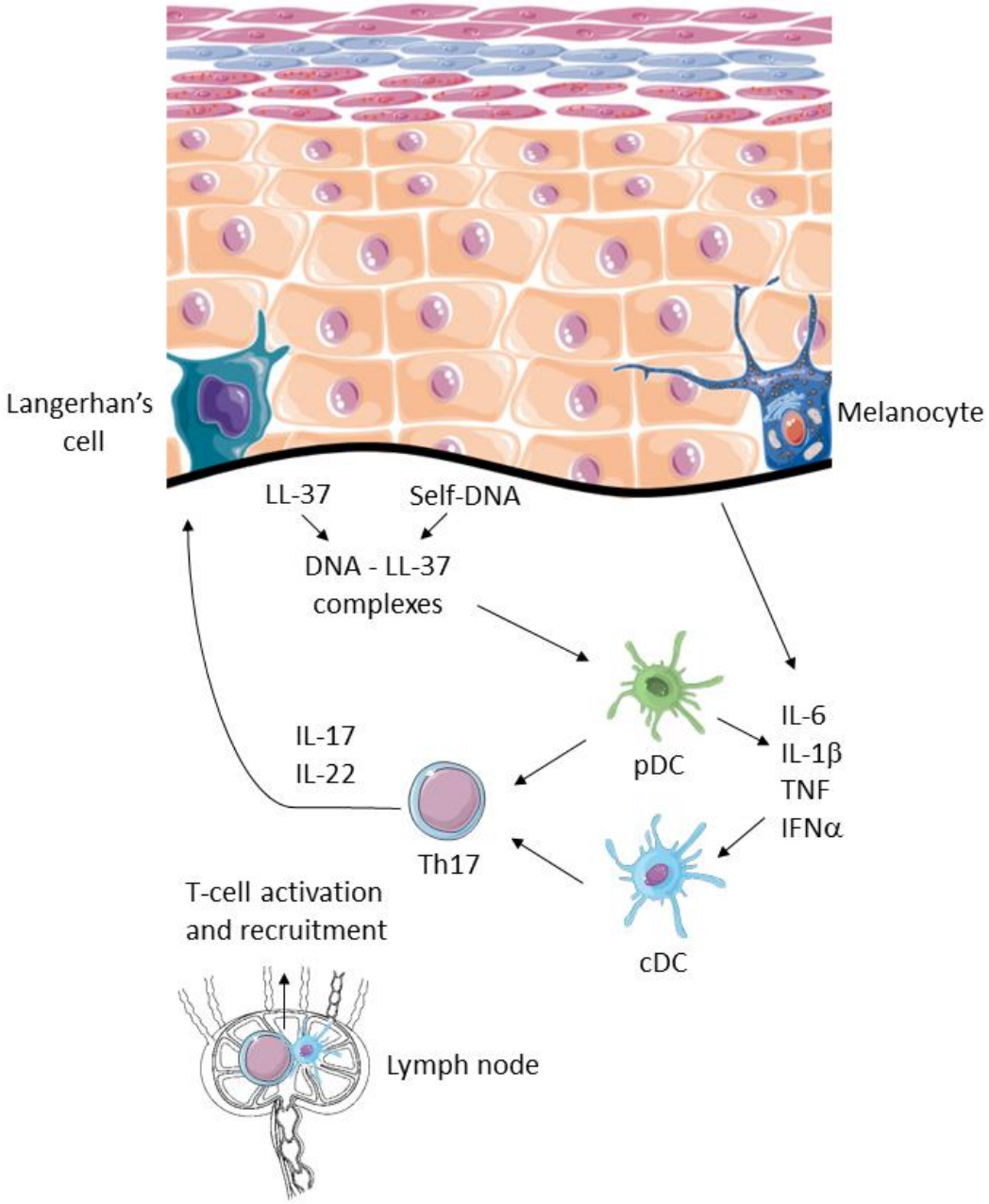


Figure 1.4| Initiation of psoriasis immunopathogenesis

Graphical representation of a psoriasis immunopathogenesis initiation mechanism, driven by the release of LL-37 and self-nucleic acids by genetically pre-disposed, stressed keratinocytes. The LL-37/self-DNA complex can induce an uncontrolled production of type 1 IFN by plasmacytoid dendritic cells (pDCs) within the skin. IFN is a potent regulator of myeloid DC (mDC) and T cell activation and maturation. In conjugation with IFN, other pro-inflammatory cytokines, such as IL-6, IL-1, TNF, function to activate mDCs at the lesion site. mDC-derived TNF, IL-6 and IL-20 further aggravate inflammation and hyperplasia, respectively, whereas IL-12 and IL-23 aid T cell differentiation. Depending on the mDC subpopulation, T cell activation can occur either locally or within a draining lymph node. Original figure, visual graphics taken from SmartServier.

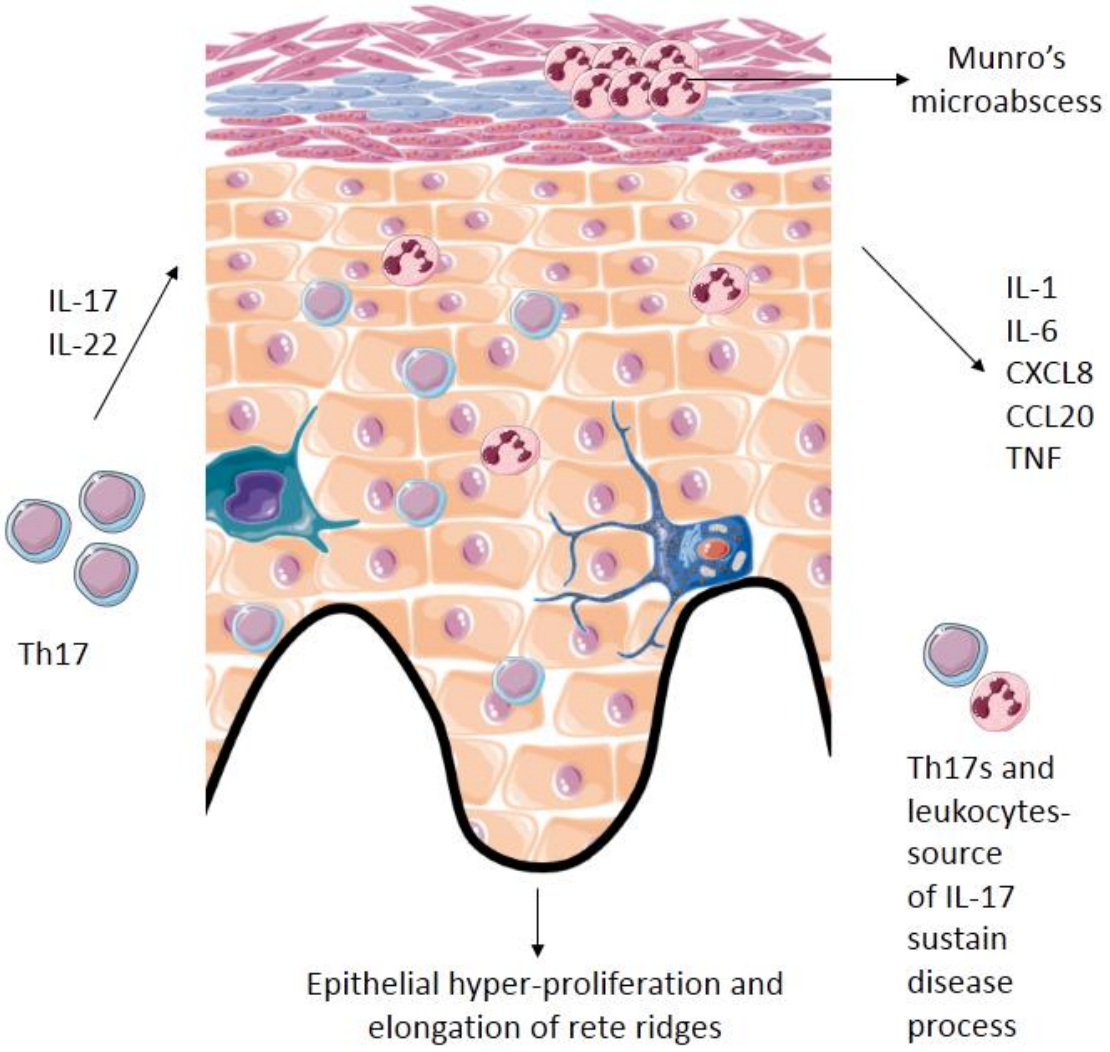


Figure 1.5| Propagation of psoriasis immunopathogenesis

Graphical representation of the establishment and amplification of pro-inflammatory loops in psoriasis immunopathogenesis, following initiation (Figure 1.4). The activation of keratinocytes by IL-17, IL-22 and other pro-inflammatory cytokines leads to the amplification of multiple pathogenic positive feedback loops, such as the IL-17/IL-23 loop. This culminates in the recruitment of more pro-inflammatory cells (e.g. neutrophils), epidermal infiltration of immune cells and keratinocyte hyperplasia. Original figure, visual graphics taken from SmartServier.

1.5.6.1 Neutrophils

Neutrophils are the most abundant leukocytes in the human blood, representing 70% of all circulating leukocytes[395]. Neutrophils are formed in the bone marrow during haematopoiesis and their production can be enhanced during times of inflammation[395]. Neutrophils are granulocytes and are often referred to as 'first line of defence' cells, as they arrive early at the site of inflammation. When activated neutrophils can employ a number of potent defence mechanisms, including phagocytosis and degranulation[396]. Their strong inflammatory properties help protect against opportunistic pathogens, but can also be harmful in autoimmune conditions[396,397].

Neutrophils are equipped with multiple mechanisms to provide defence against various pathogens. The activation of these mechanisms is dependent on neutrophil receptor expression. Neutrophils are equipped with PRRs, such as TLRs 1, 2, 4 and 9 to detect PAMPs[408]. Engagement of neutrophil TLRs has been shown to slow neutrophil apoptosis and prime neutrophils for enhanced responses[408]. Neutrophil cytokine and chemokine receptors (GPCRs) regulate the level of neutrophil activation, migration and cytokine production[410]. Neutrophils achieve microbial clearance by employing several mechanisms to neutralise potentially harmful pathogens, including phagocytosis, reactive oxygen species (ROS) generation and utilisation of their potent granules.

Neutrophil granules are particularly important because they contain extremely potent enzymes and mediators for killing pathogens. On the basis of granule content and extent of mobilisation, three main different types of neutrophil granules are now recognised[412]. Granules, which contain peroxidase are known as azurophilic/primary, whereas peroxidase-negative granules are further subdivided on the basis of relative lactoferrin (specific granules) and gelatinase (gelatinase granules) content[412,413]. Neutrophils also contain highly mobilisable specific vesicles, abundant in plasma membrane receptors, such as CD14, CR3, CR1, FcγRs[414]. All granules store antibacterial lysozyme, whereas nicotinamide adenine dinucleotide phosphate (NADPH) oxidase components can be found in specific and gelatinase granules[417]. Importantly, myeloperoxidase and NADPH oxidase participate in ROS generation, a process critical for pathogen killing[418]. Defects in NADPH subunits in humans leads to the development of chronic granulomatous disease (CGD), highlighting the importance of ROS generation in pathogen killing[419]. However, it is still incompletely understood how ROS kill pathogens. . Importantly, neutrophil degranulation can be triggered during activation and can lead to the release of the

aforementioned potent mediators into the local microenvironment[421]. Although this is an anti-microbial mechanism, it has been implicated as a tissue damaging process in inflammatory conditions[422]. Neutrophils can store and produce a plethora of pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-17, TNF, G-CSF, IFN, VEGF) and chemokines (e.g. CCL20, CXCL8, CXCL10), allowing them to activate, stimulate, and recruit more pro-inflammatory cells[423,424].

A newly described antimicrobial neutrophil defence mechanisms involves the extracellular release of decondensed chromatin and granular contents. This process is associated with cell death and has been termed NETosis, from Neutrophil extracellular traps. NETosis provides protection by trapping pathogens within the released chromatin and exposing pathogens to antimicrobial peptides (e.g. LL-37, S100A) and enzymes (myeloperoxidase, neutrophil elastase)[425]Importantly, NETosis can be enhanced by the presence of pro-inflammatory mediators, such as CXCL8, TNF, PAMPs, IL-1[430,431]. This suggests that neutrophils are more likely to induce NET release in a pro-inflammatory setting, present in autoimmune conditions. This in combination with the highly immunogenic nature of DNA-AMP complexes, makes NETosis a potentially detrimental process in autoimmunity.

Neutrophil-derived mediators can also have a regulatory effect on surrounding cells. For example, lactoferrin, chemokines, and defensins can be highly chemoattractive to DCs, whereas direct cell-to-cell interactions can stimulate DC maturation[433]. This, in turn, can lead to local T-cell activation by neutrophil-primed DCs and enhance inflammation. Further to this, NET-released LL-37 and self-DNA can form complexes, which can activate plasmacytoid DCs, triggering the activation of potentially self-reactive T-cells[434]. Neutrophils also express low levels of MHC class II and co-stimulatory molecules, suggesting they might directly interact with CD4+ T-cells and present antigens[435]. Moreover, a subpopulation of antigen-carrying neutrophils, expressing CCR7, has been shown to be able to migrate to lymph nodes[437]. Such neutrophils could either directly engage in antigen presentation to T-cells or deliver the antigens to DCs for T-cell presentation.

Therefore, neutrophils are equipped with various potent pro-inflammatory defence mechanisms, making them one of the most important immune system components. However, the dysregulation or apparent activation of neutrophils and adjacent mediators could cause pathologic inflammation in conditions, such as autoimmune diseases.

1.5.6.2 Dendritic cells

DCs are professional antigen presenting cells (APCs), which can induce primary immune responses[438]. DCs can integrate information from infection or tissue damage sites and present processed antigens to naïve T-cells in secondary lymphoid organs or locally. Antigen processing is associated with DC maturation and acquisition of properties required for the coordination and regulation of T-cell responses[438]. DCs provide vital soluble and cell-bound signals to naïve T-cells, directing their differentiation and subsequently creating a pathogen-specific immune response (or indeed, a Th1/Th2/Th17 skewed disease state). DCs are also important for the orchestration of pathologic responses in inflammatory diseases, such as psoriasis. For example, it is believed that DCs play a key role in Th17-skewed immune responses in psoriasis[726]. The role of DCs in psoriasis immunopathogenesis will be discussed in further detail in chapter 1.5.9.2.

DC populations are highly heterogeneous in terms of phenotype and function and their classification has been challenging. DCs are now classified on the basis of their ontogeny and related function[439]. The majority of dendritic cells arise in the bone marrow from a common macrophage/DC progenitor, derived from a common myeloid hematopoietic stem cell progenitor[440]. The common macrophage/DC progenitor will give rise to the common DC precursor and subsequently conventional DCs (cDCs) and pDCs[440]. Furthermore, DCs can also arise from monocytes upon differentiation in the tissues. Such monocyte-derived DCs and plasmacytoid DCs make up the non-conventional DC population[441]. The majority of the information regarding DCs comes from animal studies, due to tissue accessibility.

The main function of cDCs is to prime T-cell responses, therefore cDCs have evolved mechanisms that make them extremely efficient at capturing antigens. Antigen capture is achieved via various methods, such as macropinocytosis, receptor-mediated pinocytosis (via C-type lectin and Fcγ receptors), and phagocytosis[442-445]. On the basis of antigen accessibility, cDCs can be divided into lymph node (LN)-resident or migratory[446]. The migratory pattern of cDC depends on chemokine receptor expression, but generally those cells acquire antigens in the periphery and migrate to a draining lymph node to prime T-cells[446]. Upon antigen capture the dendritic cell enters a maturation phase, which is further stimulated by PAMPS and cytokines (e.g. TNF, IL-6, IL-1)[449]. The maturation of cDCs is associated with up-regulated surface expression of LN-homing receptors (e.g. CCR7); T-cell co-stimulatory molecules (e.g. CD40, CD58, CD80, CD86); MHC class II molecules. During DC maturation, chemokine receptors, which allow tissue-specific access, such as CCR2 and CCR6, become down-regulated, limiting cytokine responsiveness[450-453]. Changes in morphology, allowing high cell motility, also occur during this process[452]. CCR7

expression is critical for LN-homing, as it allows DCs to follow CCL19/CCL21 gradients to the lymph nodes[446,453]. In the LN, DCs become located at the T-cell zones in a CCR7-dependent manner[454]. It has been shown, that CCR7 deficient mice have abrogated DC homing to LNs[454]. In order to generate CD4+ T-cell responses, DCs load the acquired exogenous antigens onto MHC class II molecules[455]. MHC class II molecules are usually expressed by professional APCs. Conversely, the generation of CD8+ T-cell responses is dependent on the loading of ligands onto MHC class I molecules; however, the more ubiquitously expressed MHC class I molecules load endogenous antigens derived from proteins synthesised within the cells. Importantly, it has been demonstrated that DC MHC class I molecules can also load exogenous antigens in a process known as cross-presentation[456]. Therefore, DCs have evolved pathways which allow them to stimulate various T-cell responses, including CD8+ T-cell responses[457].

On the basis of development, function, and cell surface molecule expression two main cDC subsets are recognised, namely cDC type 1 (cDC1) and cDC type 2 (cDC2)[458,459]. For example, in mice, cDC1 are the primary DC subset that carries out CD8+ T-cell activation via cross-presentation[460-462]. However, data suggest that cross-presentation is not DC subset restricted in mice and human[463,464]. cDC2 have been associated with the priming of CD4+ T-cell responses[464-466]. DCs can provide CD4+ T-cells with a distinct cytokine profile to stimulate T-cell differentiation into the phenotype most appropriate for insult elimination[467]. Furthermore, the DC phenotype and maturation level can influence the induction of T-cell differentiation towards a specific phenotype[449,468]. It is important to note that monocyte-derived DCs can also prime CD4+ T-cell responses[469]. It remains unclear what prompts the 'division of labour' between cDC1 and cDC2 populations. Mouse cDC1 and cDC2 populations express CD103 and CD11b, respectively, whereas human cDC1 and cDC2 populations are characterised by CD141 and CD1c expression, respectively[470,471].

pDC produce high levels of IFN-α/β upon TLR7/9 stimulation and are therefore important for fighting viral infections[472,473,540]. pDC-derived cytokines, in combination with other pro-inflammatory cytokines, can induce activation of cDC maturation and stimulation of leukocytes[474,475]. pDCs can also produce chemokines and induce further leukocyte recruitment[476]. pDCs are absent from steady-state skin but can be recruited during times of inflammation[539]. These cells are poor at antigen presentation; however, their ability to produce pro-inflammatory mediators has been implicated in the pathogenesis of autoimmune conditions, such as psoriasis[540].

1.5.6.3 T-cells

T-cell lymphocytes serve as primary effectors for cell-mediated immune responses. T-cells use a highly specialised T-cell receptor (TCR) to recognise foreign antigens presented in the context of an MHC molecule[477]. The TCR allows T-cells to recognise a broad range of antigens from pathogens, tumours, and the environment. These cells are critically important for the establishment and maintenance of immune memory, self-tolerance, and homeostasis[478]. T-cells have been implicated as important drivers of inflammation in autoimmune and other inflammatory conditions, as evidenced by the efficacy of T-cell directed therapies in many of these conditions[479].

T-cells originate from common lymphoid progenitors in the bone marrow, which migrate to the thymus early during T-cell development. Upon arrival to the thymus, the T-cell progenitors have a double negative ($CD4^-CD8^-$) expression profile[480-483]. During the maturation process thymocytes commit to either the $\alpha\beta$ or $\gamma\delta$ T-cell lineage, which refers to the type of TCR the T-cell will express[482]. What determines T-cell commitment to either lineage is not completely understood. Studies suggest that the signal strength transduced by the TCR, during TCR rearrangement, determines lineage commitment (weak signal denotes $\alpha\beta$ fate, whereas a strong signal - $\gamma\delta$ fate)[484]. During TCR gene re-arrangement, in $\alpha\beta$ T-cells, the TCR and its specificity are generated, yielding a double positive (DP) ($CD4^+CD8^+$) antigen-specific T-cell[485,486]. DP cells undergo positive selection to test ability to bind antigen in the context of self-MHC. T-cells unable to recognise antigen-MHC complexes are eliminated (approximately 95% of developing T-cells) via induced apoptosis[487-489]. T-cells selected on MHC class I acquire a CD8+ phenotype, whereas MHC class II selection yields a CD4+ T-cell expression profile[490-492]. This is important as T-cell ability to recognise antigen in the context of a specific MHC molecule influences T-cell function. Single positive cells undergo a process of negative selection, whereby T-cells with high affinity TCR to self-MHC are eliminated[493]. This is important as it limits the release of possibly autoreactive T-cells into the periphery. $\gamma\delta$ T-cell do not undergo positive or negative selection, but ligand interactions in the thymus pre-determine their effector functions[494]. These processes are dependent on thymus-resident specialised stromal cells and the cytokines and chemokines these cells produce.

Upon thymic egress, naïve $\alpha\beta$ T-cells have a CD45RA+CCR7+CXCR4+ phenotype and are mainly targeted to secondary lymphoid organs and circulation[495] In the LNs naïve T-cells are

targeted to specific zones, where they co-localise with dendritic cells, and scan the microenvironment. [454,498]. Naïve T-cells are unable to produce an effector response and require activation in order to provide protection against pathogens. The activation of naïve T-cells is a process, dependent on coordinated T-cell-Antigen presenting cell (APC) interactions and provision of three distinct signals to T-cells[455,456,467]. The first T-cell activation signal involves the recognition of a specific antigen, in the context of an appropriate MHC molecule, by the TCR[499]. Depending on the $\alpha\beta$ T-cell type, the interactions between T-cells and APCs are further stabilised by either CD4 or CD8. CD4 and CD8 are TCR co-receptors, which bind to either MHC class II, or class I molecules, respectively (Figure 1.6)[501]. Therefore, the expression of a co-receptor limits and determines T-cell interactions with APCs. Co-stimulatory and adhesion molecules are recruited to the site of TCR-MHC interaction, termed the immunological synapse[502]. The co-stimulatory molecules provide the second signal in the T-cell activation cascade. For example, CD28 is a co-stimulatory molecule, which binds to its ligands (CD80 and CD86) on APCs to mediate T-cell activation[503]. Importantly, co-stimulation can also fine tune T-cell activation by TCR signal inhibition. For example, the TCR signal inhibiting cytotoxic T lymphocyte antigen (CTLA)-4 acts as a CD28 competing receptor for CD80/CD86 binding[507].

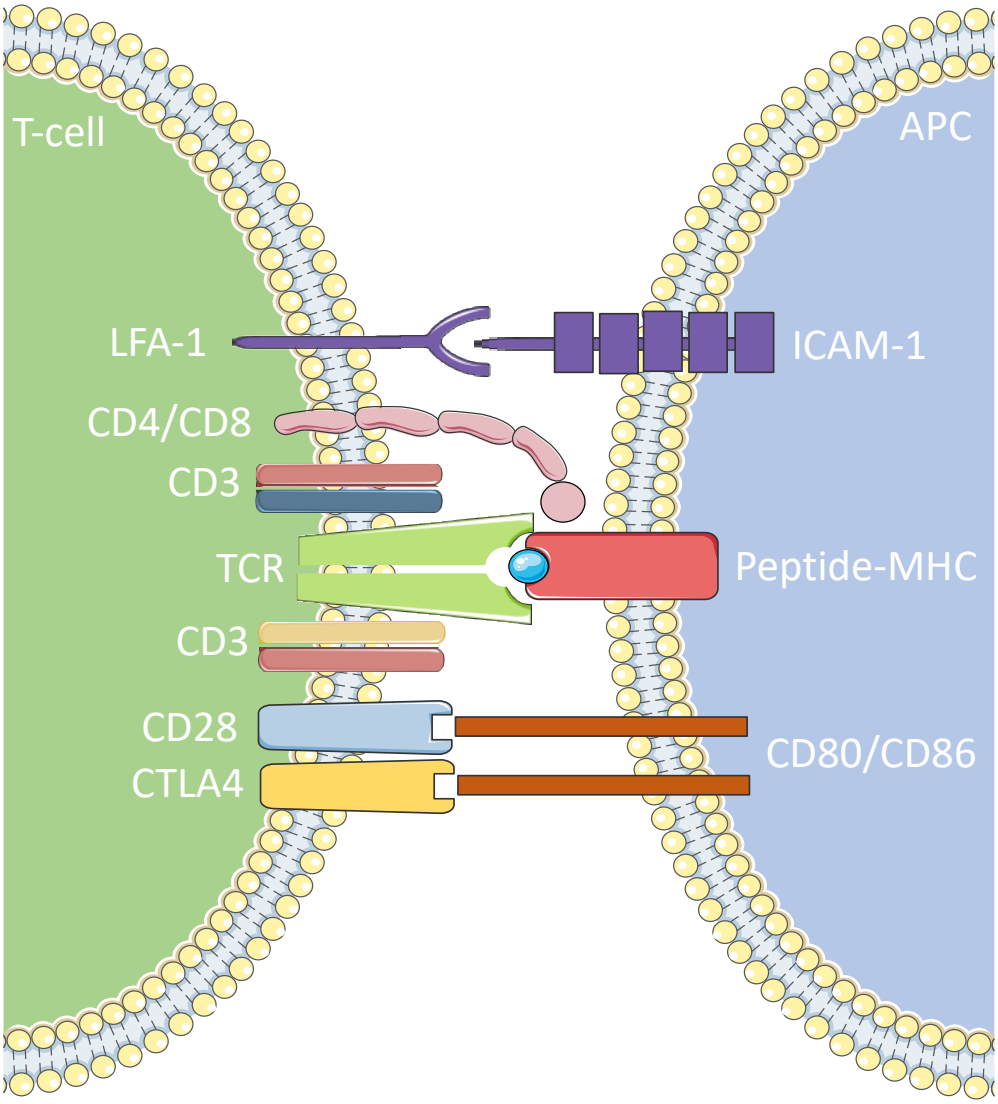


Figure 1.6| T-cell-Antigen presenting cell interactions, during T-cell activation

Graphical representation of the molecular interactions, which take place during the activation of T-cells, between a T-cell and an antigen presenting cell. The T-cell recognises an antigen, presented in the context of an MHC molecule, via its receptor and receives co-stimulatory signals. Multiple molecular interactions support these events, including lymphocyte function-associated antigen 1 *(LFA-1)/ Intercellular Adhesion Molecule 1 (ICAM-1); CD28/CD80; CD28/CD86; CTLA4/CD80; CTLA/CD86. Original figure, vector components obtained from SmartServier.*

The third signal refers to the stimulation of activated T-cells by APC-derived soluble or membrane bound cytokines[467,509-511]. The cytokine milieu produced by the antigen-bearing APCs is specific and pathogen-dependent[512-515]. The molecular integration of these cytokine signals can induce uncommitted T-cells into different effector subsets. Differentiated T-cell effector subsets can utilise mechanisms to promote immune responses suited to the invading pathogen. Inducible and specialised T-cell responses have evolved as a response to the largely diverse spectrum of opportunistic pathogens. Once activated, T-cells downregulated the LN-homing CCR7 and upregulate pro-inflammatory chemokine receptors, which allow them to leave the LNs[516].

1.5.6.3.1 Helper T-cell responses

T-cell effector functions have been broadly categorised as either 'helper' or 'cytotoxic'. Helper T-cells are CD4+ and can produce a variety of cytokines and chemokines to induce particular immune responses[541]. This is usually achieved by stimulating neighbouring cells to produce pro-inflammatory mediators. CD4+ T-cells are classified into several distinct groups based on the cytokine profile they produce. These groups include T helper (Th)1, Th2, Th17, Tfh, Treg cells[541-544,547]. As discussed, the generation of these CD4+ T-cell subtypes is dependent on distinct APC-derived cytokines (Figure 1.7), produced in response to invading pathogens. The cytokine signals induce a transcription factor programme to drive T-cell differentiation into a specific phenotype, capable of producing a distinct cytokine milieu (Figure 1.7). For example, intracellular pathogens prompt IFN-α/β and IL-12 production by APCs[545]. This set of cytokines triggers expression of the transcription factor (TF) T-bet, which drives Th1 differentiation[546]. Th1 cells can produce a robust amount of IFNγ and IL-2, which can activate local cells, such as macrophage, and induce phagocytosis[548,549]. It has been shown that mice which lack T-bet have diminished Th1 responses (e.g. low IFNγ levels) and are susceptible to *M. Tuberculosis* infections[550]. Conversely, extracellular pathogens promote Th2 responses, which induce the activation of cells that specialise in parasite elimination (e.g. eosinophils, mast cells, basophils)[551-553]. Th17 cells have, in addition to a key pathogenic role in psoriasis, also been implicated in fighting extracellular pathogens, such as bacteria and fungi[554a]; however, data suggest that they might also provide protection against intracellular insults[554,555]. It is important to mention that T-cell differentiation is not always induced in response to a particular pathogen. For example, Tfh cells are induced in support of immune responses commonly mounted against

various infections[547]. Tfh cells promote high-affinity antibody generation in the B-cell germinal centre reaction by providing important co-stimulation and cytokine signals[547]. Mice lacking Tfh cells fail to develop high affinity antibodies and are therefore more susceptible to infections[556]. Furthermore, other T-cell phenotypes can also participate in directing the antibody responses. For example, Th2 cells produce cytokines which stimulate class-switching to IgE and IgA, associated with anti-parasitic responses[557,558]. T-cell responses are also mounted to regulate the strength of the immune responses. Tregs which arise in the thymus, from thymocytes, are called natural Tregs (nTregs), whereas Tregs which arise in the periphery, from conventional T-cells, are called induced Trgs (iTregs)[544,559]. Regardless of their origins, Tregs are defined by the expression of the transcription factor forkhead box P3 (FOXP3) and CD25 (the α chain of the IL-2 receptor)[544]. Treg cells can produce cytokines, such as IL-10 and TGF-β which inhibit cell proliferation and pro-inflammatory cytokine production[544,559]. Further to this, Tregs can also express the inhibitory co-stimulation molecule CTLA-4 and thus limit T-cell activation[560]. Their functional impairment has been associated with autoimmunity in humans. Further to this, mice which lack Treg cells develop severe multiorgan autoimmune conditions[561]. Therefore, the different T-cell helper subtypes possess unique features which allow them to fight specific pathogens and in accordance with their function. Further to this, the different Th subsets are also defined by the expression of different sets of chemokine receptors, which allow appropriate, tissue-specific migration. For example, Th1 cells express CCR5, CXCR3 and CXCR6, Th17 cells express CCR6, and Th2 cells - CCR2, CCR8, CX3CR1[562,563]. All of these features work together to facilitate pathogen eradication and can be modulated by microenvironmental stimuli when needed.

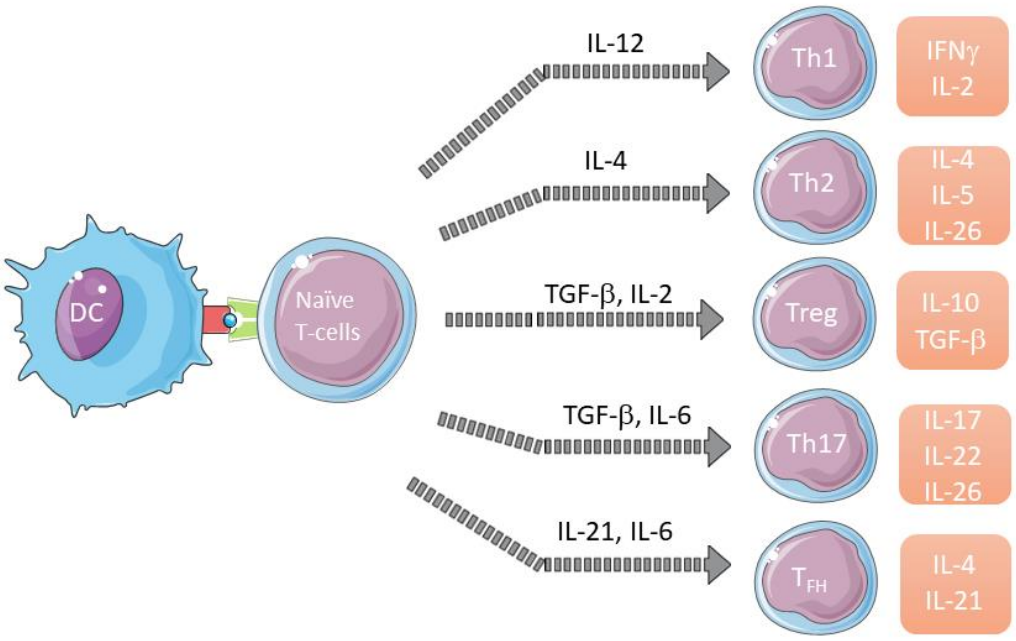


Figure 1.7 | Helper T-cell differentiation and cytokine production profile.

A graphical representation of the cytokines required for the differentiation of specific helper T-cell phenotypes and the cytokine profile they produce. Following antigen recognition and co-stimulation, the naïve T-cell is provided with differentiation signals, in the form of cytokines, by the antigen presenting cell. This engages a specific, to the helper T-cell phenotype required for pathogen clearance, transcription factor programme, which guides the differentiation process. The resultant helper T-cell can produce specific cytokines upon activation and differentiation. Original figure, visual graphics taken from SmartServier.

Crucially, a great number of immunological conditions, such as allergies and autoimmune disorders, are T-cell mediated. The dysregulation of T-cell responses can therefore be detrimental to health. Th2 cells are known drivers of allergies, whereas Th1 and Th17 cells have been implicated in multiple autoimmune conditions, such as psoriasis, but also Crohn's disease and psoriatic arthirits[564]. As the experiments in this thesis focus primarily on T-cells of a Th17 phenotype, Th17 cells will be discussed in more detail.

1.5.6.3.2 Memory T-cell responses

Upon pathogen elimination, T-cell populations decline, and only small populations of 'memory' T-cells remain. The memory phenotype commitment and survival are also mediated by cytokine cues (e.g. IL-7, IL-15) and specific transcription factors (B lymphocyte-induced maturation protein-1 (blimp-1), B-cell lymphoma 6 protein (bcl-6))[601-604]. Both CD4+ and CD8+ T-cells can acquire a memory phenotype. These cells have participated in the primary immune responses and have specific characteristics. For example, memory T-cells are easy to activate, proliferate rapidly, and can elicit quick effector responses[605]. Memory T-cells can access secondary lymphoid organs, peripheral tissues and can acquire a resident phenotype at the site of primary infection[606-608]. They can provide quicker responses should a reinfection by the same pathogen occur[605]. Two main memory T-cell subtypes exist – central memory and effector memory T-cells. Central memory T-cells express high levels of CCR7 and IL-7R, which allows them to traffic between secondary lymphoid organs and proliferate upon antigen re-encounter[609]. Therefore, they can replenish the infection site with new effector cells if needed. Effector memory T-cells express no CCR7 and low IL-7R levels and have low proliferative abilities and do not home to secondary lymphoid organs[609]. Effector memory T-cells traffic through the periphery and have rapid effector functions, meaning they produce granzyme B and pro-inflammatory cytokines, such as IFN-γ. Therefore, effector memory T-cells provide quick and immediate protection upon antigen re-encounter, which can later be supplemented by effector cells derived from central memory T-cells[609]. In contrast, the heightened state of responsiveness of memory T-cells has been shown to be strongly pathogenic in inflammatory conditions, such as psoriasis[785]. Memory T-cells have not only been identified in psoriasis[785] but are believed to be involved in disease initiation and relapse[785,786]. The role of memory T-cells in psoriasis pathogenesis will be discussed in further detail in chapter 1.5.9.3.2.

1.5.6.3.3 T-cells in the skin

Human skin T-cells amount to 20 billion cells (twice as much as circulating T-cells) and 80% of those lack CCR7/L-selectin expression, signifying an effector memory phenotype[691]. The epidermis is densely populated by leukocytes, including αβ T-cells, γδ T-cells[691,692]. The dermis is not as rich in leukocytes, but is abundant in extracellular matrix, which supports leukocyte migration. The majority of αβ T-cells within the human skin are of a CD4+ memory phenotype; however, CD8+ T-cells are also present in the skin[691,693]. These cells can establish themselves into non-recirculating skin-resident populations (tissue-resident memory T-cells (Trms)) or can survey the tissue and return to circulation[694,695]. Trm cells were first discovered in psoriasis studies, whereby human non-lesional psoriatic skin was transplanted onto immunodeficient mice (the AGR129 xenotransplantation model)[698]. T-cells from the graft were not detected in the circulation and their migration into the epidermis was sufficient for plaque development, demonstrating skin confinement. Furthermore, alemtuzumab treatment in humans led to the depletion of all circulating, but not skin-confined, T-cells[694]. These cells were capable of providing protection against infection, demonstrating their immune properties. Trms can be found both in the dermis and epidermis of human skin, albeit at different frequencies. It has been shown that most of the Trms in human skin reside within the dermis, and differ from Trms in the epidermis. For example, CD103+ Trms (both CD4+ and CD8+) are enriched in the epidermis (twice as many epidermal than dermal Trms express CD103), as CD103 allows E-cadherin binding[699,700]. Furthermore, CD103+ Trms have more potent cytokine production effector functions and lower proliferative capabilities when compared to CD103- Trms. Trms can become rapidly activated upon antigen re-encounter and trigger innate and adaptive immune responses[699]. For example, CD69+ Trms have been shown to produce cytokines, such as IL-17, IL-22, IL-10, indicating that they can trigger and regulate pro-inflammatory responses[699]. Furthermore, Trms can also produce IFNα to aid DC maturation and IFNγ to recruit circulating skin-tropic memory T-cells. It has been demonstrated that recirculating skin-tropic memory T-cells elicit less potent effector responses than skin Trms[699]. HSV-1 studies in mice suggest that most of the CD8+ Trm cells are confined within the epidermis and aggregate at the original sites of inflammation[696]. Conversely, CD4+ Trm cells were shown to be primarily targeted to the dermis[696]. Studies in mice, whereby Kaede transgenic mouse skin was exposed to violet light, to allow tracking of cutaneous T cells, demonstrated that a portion of the skin-associated CD4+ T-cells can return to circulation[695]. It has been suggested that the recirculating CD4+ T-cells, that

retain ability to re-enter skin, could be of a distinct phenotype, different to T effector memory cells. These findings suggest that the skin contains unique resident memory T-cell populations, which provide local protection against infection, but can also act to recruit additional recirculating T-cells. The exact role of resident T-cells in local immunity and autoimmune inflammation remains to be clarified.

γδ T-cells contribute to only a small portion of the T-cells in the human epidermis. In contrast, γδ T-cells in mice represent the bulk of epidermal T-cells and form a tight network[701,702]. Epidermal γδ T-cells contribute to wound healing, in both human and mice, but they can also participate in the inflammatory process[701,702]. Mice lacking γδ T-cells exhibit reduced inflammation, slow keratinocyte proliferation and wound healing[704]. Dermal γδ T-cells in mice have been shown to promote CD4+ T-cell immunity, by a yet unknown mechanism, and induce neutrophil recruitment via IL-17 production[703]. The understanding of γδ T-cells in human skin immunity is very limited, but their importance in autoimmune conditions has been previously highlighted.

1.5.6.4 Skin-Associated Lymphoid Tissue (SALT)

As discussed earlier, the skin provides rapid and efficient protection against various opportunistic pathogens by utilising multiple barriers. The immune cell barrier of the skin is facilitated by the coordinated action of innate and adaptive leukocytes, which patrol or reside within the skin, and keratinocytes[519,535,691,692]. The controlled interactions between leukocytes, keratinocytes and antigens allows for the processing of the antigenic signal and activation of appropriate immune responses, if needed[705]. Constitutive and inducible immune pathways, therefore, govern the eradication of opportunistic pathogens and cutaneous neoplasms, whilst maintaining a state of immune tolerance. However, in autoimmune conditions, such as psoriasis, these background immune circuits can become amplified and cause pathology[705,707]. The cutaneous immune compartment is known as skin-associated lymphoid tissue (SALT)[706].

The main function of keratinocytes is to replenish the stratum corneum and perform mechanical barrier functions. However, keratinocytes are also a component of the innate immune system. Keratinocytes express a multitude of PRRs and can therefore recognise exogenous and endogenous antigens[708]. TLRs help keratinocytes detect PAMPs, such as lipopolysaccharide

(TLR4), flagellin (TLR5), nucleic acids (TLR3/7/8/9)[708]. NLRs (e.g. Nod1 and Nod2) recognise bacterial peptidoglycan, viral, fungal, and self-antigens, whereas C-type lectins detect fungal PAMPs[709]. Keratinocytes can also release (via the inflammasome complex) and sense endogenous damage-associated molecular patterns (e.g. ATP, heat shock protein), produced from stressed or dying host cells[708-710]. These and more sensing mechanisms allow a quick keratinocyte-driven response to changes in the microenvironment. The elicited response may involve the production of pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF, IL-10, IL-33)[709] and chemokines (e.g. CXCL8, CXCL9, CXCL10, CXCL11, CCL20)[295]. Furthermore, keratinocytes are also known to constitutively produce small amounts of antimicrobial peptides (AMPs), such as β-defensins and cathelicidins[711]; however, AMP production becomes elevated during inflammation[713]. AMPs are essential for the host defence against infections and can directly kill pathogens, recruit leukocytes and enhance inflammation. Other microbicide produced by keratinocytes include, ribonucleases (RNases) and S100 family members[712,714]. Therefore, the keratinocyte response to PAMPs and DAMPs is essential for the activation and recruitment of leukocytes to the skin.

1.5.7 Transmigration

The immune responses mounted against pathogens involves the recruitment of leukocytes to the infection site. Leukocyte recruitment is facilitated by chemokines, which become upregulated during times of infection, in a multi-step process known as TEM[616]. During the TEM cascade, leukocytes in the circulation recognise the activated vascular endothelium in inflamed tissue and traverse through the blood vessels. These interactions happen in a well-orchestrated manner through a series of steps, namely capturing, rolling, leukocyte arrest, crawling and transmigration (Figure)[616].

A number of adhesion molecules facilitate TEM and are critical for the completion of the cascade, as evidenced by antibody neutralisation and genetic deletion studies. For example, the rolling step of the TEM cascade is mediated by molecules, known as selectins and their adjacent receptors[617,618]. E-selectins and P-selectins are expressed by inflamed endothelial cells and interact with ligands, such as P-selectin glycoprotein ligand 1 (PSGL1), cutaneous lymphocyte-associated antigen (CLA), expressed by leukocytes[617,618]. These interactions have very high on- and off- rates, allowing leukocytes to bind to the inflamed endothelium under conditions of blood-flow[619,620]. Moreover, studies suggest that the shear force, created by the blood-flow, is required for selectin-driven adhesion, as leukocyte rolling halts upon blood-flow seizure[621,622].

Leukocyte slowing down is also supported by the elongation of rear tethers and adhesive substrates, such as slings, by the leukocyte itself[623]. This allows leukocytes to sample the endothelial surface and if no stimulus is detected, leukocytes detach and return to circulation. In vivo studies have shown that leukocyte arrest and firm adhesion are triggered by leukocyte interactions with chemokines[624]. As discussed earlier, chemokines can be produced by the endothelium or at a distant site. Importantly, distantly produced chemokines can be transported and anchored to the luminal surface of the endothelium via ACKR-mediated transcytosis[342]. GAGs are also responsible for the immobilisation of chemokines at the endothelium during this cascade[625].

The chemokines that come into contact with rolling leukocytes trigger an event known as 'inside-out' signalling[626-628]. This leads to the instantaneous activation of leukocyte proteins, known as integrins. Inside-out signalling changes integrin conformation from low- to high-affinity, leading to enhanced ligand binding properties[626,627]. This is an important event, as it allows integrins to develop high avidity (overall strength of binding) and affinity (binding strength at a single binding site) for their respective ligands and establish firm adhesion[627]. Integrins, such as lymphocyte function-associated antigen 1 (LFA-1), αMβ2, α1β1, αXβ2 are expressed by leukocytes at varying degrees (e.g. LFA-1 is expressed by all leukocytes, whereas αMβ2 is predominantly expressed by myeloid cells)[629,630]. Their endothelium-expressed ligands are from the immunoglobulin superfamily and include (Intercellular Adhesion Molecule)-1–5, vascular cell adhesion molecule-1 and mucosal vascular addressin cell adhesion molecule-1[631]. Chemokine binding allows for the millisecond upregulation of integrins, subsequent ligand binding and cease of leukocyte rolling[626-628]. Leukocytes that have been brought to halt crawl on the blood vessel wall to find exit sites, such as cell junctions, from the blood vessel[632]. Crawling is mediated via αMβ2- *Intercellular Adhesion Molecule 1 (*ICAM1) interactions and can be carried out in directions opposite of the blood flow, allowing optimal scanning[632]. Disruption of such interactions during neutrophil TEM, prevents neutrophils from crawling and transmigrating[632].

Endothelial cells can form clusters of ICAM1 and Vascular cell adhesion protein 1 (VCAM1) molecules at their apical site, creating membrane extensions known as 'docking structures'[633,634]. The so-called docking structures are induced by integrin/integrin ligand interactions and are important for the initiation of the final steps in TEM. Transmigration happens at the endothelial cell junctions and relies on the reduction of contact between endothelial cells and redistribution of junctional molecules[635]. Studies suggest that induction of Rho-dependent signalling and second messenger molecules (e.g. Calcium $(Ca)^{2+}$) via VCAM1 and ICAM1 cross-linking on endothelial cells, supports the opening of inter-endothelial contacts[636-638]. Both intracellular Ca^{2+} increase

and Rho signalling have been associated with the stimulation of myosin light-chain kinase (MLCK) activity[635]. MLCK induces myosin fibre contraction, which helps endothelial cells separate[635-638]. Multiple junctional molecules, such as platelet endothelial cell adhesion molecule-1 (PECAM-1), CD99, ICAM-1-2, junctional adhesion molecule (JAM)-A-C, become engaged in various critical interactions during this process[633,639-642]. For example, homophilic PECAM-1/PECAM-1 interactions between the migrating leukocyte and endothelial cells have been shown to be important for diapedesis[639,640]. This interaction triggers kinesin-mediated microtubule-dependent membrane redistribution in the vicinity of the leukocyte, aiding its migration through the endothelium[640]. Blocking this interaction via neutralising antibodies, blocks diapedesis[640]. Furthermore, molecules which support TEM cluster at the junctions, whereas molecules that are not beneficial to leukocyte migration (e.g. vascular endothelial (VE)-cadherin) are redistributed away[643]. It is believed that the junctional molecules, which support diapedesis, are part of a complex, termed the lateral border recycling compartment (LBRC)[644]. Moreover, it has been shown that the membrane which becomes redistributed to aid leukocyte diapedesis is LBRC-derived[640,644]. Different sets of junctional molecules may become engaged during diapdesis, depending on the stimulus present. For example, IL-1β presence induces PECAM1 and JAM-A utilisation during TEM, whereas TNF and leukotriene-B_4 do not[645]. The transcellular pathway is poorly understood; however, it is believed that it relies on caveolin-1-dependent formation of intracellular channels that allow leukocyte migration through cells[646]. It has been suggested that these channels are triggered via ICAM1 interactions[646].

To complete TEM, leukocytes must migrate through the endothelial basement membrane (EBM) and pericyte sheath, which participates in EBM formation[647]. The EBM consists of collagens and vascular laminins which are interconnected in a vast network; however, points of low matrix protein (e.g. laminin-10 and collagen IV) expression in the EBM exist[647-649]. Furthermore, it has been shown that such sites co-localise with gaps in the pericyte sheath, which may be related to its role in EBM formation. Such regions of low resistance allow leukocytes to migrate more easily across those barriers[647-649]. Therefore, leukocytes are equipped with an array of molecules to complete TEM and reach the site of inflammation. It is important to note that TEM rates are increased in multiple autoimmune conditions, such as psoriasis, and contribute to tissue damage. Therefore, understanding what molecular constituents influence TEM and potentiate TEM during pathological inflammation could aid the development of anti-migration therapies.

1.5.7.1 The cAMP-signalling pathway in leukocyte migration

An important process, which is in part cAMP-regulated, in the inflammatory cascade is TEM. As discussed in the previous subchapter, TEM involves several consequent stages, which are dependent on complex molecular interactions between leukocytes and endothelial cells. Adhesion molecules (e.g. integrins, selectins) facilitate multiple such interactions and their expression is therefore critical for the successful completion of the transmigration cascade. For example, the rolling stage in TEM is selectin-dependent and has been shown to be inhibited by the action of cAMP-elevating agents[650]. Further to this, direct PDE inhibition in neutrophils has been associated with reduced chemotaxis[651]. Such studies underscore the importance of cAMP signalling in adhesion molecule expression and leukocyte migration.

The relative role of cAMP effector proteins in the regulation of leukocyte migration has been addressed previously. For example, cAMP has been shown to drive the reduction of neutrophil integrin (e.g. αMβ2) expression and subsequent block of adhesion to vascular endothelium[650]. Inhibition of the cAMP effector protein PKA protects neutrophils against this cAMP-orchestrated adhesion blockade[652,653]. This suggests that PKA controls neutrophil adhesion via modulation of integrin expression. Furthermore, PKA has been reported to inhibit the activation of RhoA, a small GTPase regulator of actin-based contractility, in neutrophils[654]. In these experiments, cAMP elevation led to RhoA reduced activity and associated reduced integrin-dependent binding (α4β1 and αMβ2) and loss of neutrophil adhesion. This was saved by selective PKA inhibition. Therefore, cAMP-PKA may affect the actin-based cytoskeleton and subsequently integrin-dependent leukocyte adhesion. The attenuation of neutrophil chemotaxis via PDE inhibition could therefore be partially explained by the outlined cAMP-PKA signalling mechanisms. Contrary to these findings, however, PKA has also been associated with enhanced αMβ2 cell surface expression, adhesion, and leukocyte migration[655,656]. It has been suggested that PKA may be important for cell polarisation, as PKA inhibition prevented lymphocyte polarisation in response to chemokine stimulation[657]. It is likely that PKA function will depend on the particular cAMP stimulus and abundance and segregation of intracellular PKA and cAMP pools.

cAMP-Epac-1 signalling has been shown to promote integrin surface expression and adhesion of monocytes and lymphocytes. This is achieved by the Epac-1-dependent stimulation of the Ras GTPases homologue Rap1 and subsequent up-regulation of β1/β2 integrins for increased adhesion[658,659]. It has been suggested that Rap1 stimulates integrin activity by working with its effector molecule, known as RAPL[660-662]. The Rap1-RAPL complex has been shown to translocate to the leading edge of the cell where it can stimulate integrin activation and subsequent cell

adhesion and motility[661,662]. Although cAMP is known to be able to activate Epac-1 in neutrophils, research has demonstrated that Epac-1-dependent stimulation of Rap1 cannot increase neutrophil β2 integrin expression[663]. Further studies have demonstrated that the attenuation of neutrophil migration by the PDE4 inhibitor roflumilast depends on Epac-1, rather than PKA, activation[664]. The duality of the cAMP system as a whole and the duality of each cAMP effector protein makes understanding their role in leukocyte migration extremely challenging. It still remains unknown how intracellular molecular distribution and different stimuli direct the utilisation of one pathway or effector molecule over another during TEM.

As discussed in this chapter, the effects of PDE inhibition and subsequent cAMP elevation on leukocyte migration has been previously demonstrated. However, it remains unclear if PDE inhibiting drugs, approved for the treatment of psoriasis, affect leukocyte migration. Even less well understood are the possible molecular mechanisms that may govern such effects (i.e. inhibition of leukocyte migration).

1.5.7.2 The role of calcium in leukocyte migration

Ca^{2+} is an important second messenger signalling molecule which participates in various parts of the immune response. For example, Ca^{2+} fluxes are induced as a result of the ligation of antigen and pattern recognition, scavenger, cytokine, chemokine, Fc receptors[665]. Therefore, Ca^{2+} has critical associations with processes, such as immune cell activation, phagocytosis, cytokine production, all of which are critical for pathogen eradication[666]. Induction of leukocyte chemotaxis and TEM is also critically dependent on Ca^{2+} [665]. The role of Ca^{2+} in the induction of leukocyte chemotaxis and the effects of disturbing the Ca^{2+} system on chemotaxis will be discussed.

Resting leukocytes have a low cytosolic Ca^{2+} concentration, when compared to the extracellular milieu[667]; however, leukocytes harbour an intracellular Ca^{2+} source in their endoplasmic reticulum (ER). The fine communication between the ER Ca^{2+} stores and membrane calcium channels facilitate the spatial and temporal regulation of leukocyte Ca^{2+} transients[668,669]. The activation of neutrophils during the TEM cascade and adhesion molecule up-regulation for cell arrest is a process mediated by the chemokine receptor binding[665]. Generally, the engagement of a GPCR and other molecules, such as FcγRs, β2-integrins, leads to the intracellular burst of Ca^{2+} [670-672]. This process is known as store-operated calcium entry (SOCE)[673]. This is achieved by the activation of phospholipase C, which leads to the cytosolic elevation of inositol

trisphosphate (IP3) and DAG[673,674]. IP3 activates its ER-bound receptor, which triggers the release of Ca^{2+} from the ER stores into the cytosol[673,674]. Most of the Ca^{2+} is transported back to the ER via the sarco(endo)plasmic Ca^{2+} reticulum ATPase (SERCA); however, some of the Ca^{2+} leaves the cell (efflux) via the plasma membrane Ca^{2+} ATPase (PMCA), rendering the ER depleted of Ca^{2+}. The ER, however, is equipped with proteins, such as stromal interaction molecule 1 (STIM1), which sense the drop in Ca^{2+} stores[675]. STIM1 can then translocate to the ER-plasma membrane (PM) junction and activate PM Ca^{2+} channels (e.g. Orai1, transient receptor potential channels), leading to a Ca^{2+} influx[675-677]. It has been suggested that these events happen in a strict chronological manner. For example, a study demonstrated that the influx of Ca^{2+} during SOCE in chemoattractant activated neutrophils starts after efflux termination[678]. Many of the findings outlined above have been achieved via short-interfering RNA-mediated knockdown of molecular participants and elegant immunofluorescence studies. It has been reported that local Ca^{2+} fluxes are important for the integrin-mediated arrest of neutrophils during TEM. During the arrest step of TEM the adhesion molecule LFA-1 assumes a high affinity state which allows it to form strong bonds with ICAM-1[680]. The tensile tension resulting from the strong molecular bonding induces an Orai1-mediated Ca^{2+} flux, which acts to strengthen adhesion by recruiting more LFA-1 clusters[679]. Failure to form strong LFA-1-ICAM1 bonds limits the intracellular Ca^{2+} flux at the site[679]. Orai1 has also been shown to induce the local Ca^{2+} transients which guide actin polymerisation during leukocyte migration[681]. Indeed, an enriched calcium influx at the leading edge of migrating neutrophils in zebra fish has been reported[682]. Furthermore, the knock-down of STIM1 has been shown to abrogate the activation of the actomyosin macromolecular complex, which is important for the generation of cell contractility. This resulted in failure of cells to migrate[683]. Furthermore, the formation of local Ca^{2+} transients has been shown to be important in more neutrophil effector functions, such as ROS production, degranulation, and phagocytosis[684]. These findings underscore the importance of the Ca^{2+} flux system in the immune responses.

Possible dysregulations in the Ca^{2+} system could have a negative effect on a number of immune effector functions. For example, it has been demonstrated that the removal of intracellular Ca^{2+} from neutrophils abolishes their ability to migrate[682,685,686]. Interestingly, the elevation of cAMP has been associated with the inhibition of Ca^{2+} fluxes and leukocyte migration[685,687-689]. As discussed earlier, cAMP levels become elevated in leukocytes as a result of treatment with PDE4 inhibitors, such as Apremilast. It has been suggested that PDE4 inhibitors supress neutrophil migration via the cAMP-dependent activation of downstream enzymes, such as PKA. Multiple PKA-driven mechanisms of migration inhibition have been proposed and are discussed in paragraph 1.5.7.1[652-657]. In addition to those, recent studies have suggested that cAMP-driven

PKA induction may also interfere with the Ca^{2+} fluxes triggered in response to chemoattractant stimulation of neutrophils[687]. According to R. Anderson et al, treatment with PDE4 inhibitors leads to the cAMP-PKA driven up-regulation of SERCA activity. The up-regulation of SERCA activity creates a competition between SERCA and PMCA for endogenous Ca^{2+}, following neutrophil stimulation with chemotactic peptide. This affects the whole SOCE cascade outlined above. For example, the ER stores become re-filled with endogenous Ca^{2+}, meant for efflux via PMCA, leading to the re-sequestration of cytosolic Ca^{2+} and limited SOCE. Unfortunately, these studies never alluded to the implications of their findings for leukocyte migration. It would therefore be important to better understand how current approved and future treatments for autoimmune conditions affect Ca^{2+}-mediated effector functions, such as migration, of leukocytes.

1.5.8 Immunopathogenesis of psoriasis

1.5.8.1 Overview

Psoriasis immunopathogenesis involves cross-talk between keratinocytes and multiple innate and adaptive immune system compartments[11,13]. The interactions between keratinocytes and inflammatory leukocytes, such as dendritic cells, neutrophils, and T-cells leads to the formation of the skin plaques that characterise psoriasis[11,13]. These inter-cell interactions are not well characterised and neither are the roles of participating cells or sequence of events. It was only a relatively recent realisation that the immune system plays a critical role in the establishment of the psoriatic phenotype[715-717]. The notion that psoriasis was a result of intrinsic defect in keratinocytes, triggering an immune response, was not disproved until the 1980s-1990s[717], with a study showing that selective T-cell apoptosis leads to lesion resolution, without affecting keratinocyte survival, suggested that this view of psoriasis pathogenesis is incomplete[109]. These results were later confirmed by other studies. For example, the high association rates between psoriasis and genes responsible for the expression of MHC I molecules, strongly suggests immune system involvement[199,200]. Further to this, the injection of pre-psoriatic skin with psoriatic leukocytes has been shown to be sufficient for plaque development[717]. Finally, the success of T-cell targeting therapies for the treatment of psoriasis highlights the role of the immune system in psoriasis pathogenesis[85-91].

The focus in the field has shifted towards the better understanding of T-cell phenotypes and T-cell triggers in psoriasis immunopathogenesis[111,112]. However, the relative contribution of different leukocytes to psoriasis pathogenesis is still to be fully elucidated. The general understanding is that extrinsic environmental factors can induce genetically predisposed keratinocytes into a stress-state[718-720,733]. Stressed keratinocytes release numerous dendritic cell-activating factors, leading to a subsequent T-cell activation, further keratinocyte stimulation and excessive leukocyte recruitment[718,719]. These events are strongly dependent on cytokine production, as evidenced by the success of cytokine targeting therapies[85-91]. For example, the Th17-derived IL-17 is central to the inflammatory cascade in psoriasis. IL-17 stimulates keratinocytes to produce a plethora of pro-inflammatory mediators, such as neutrophil-stimulating cytokines[112,721]. Therefore, cytokine production allows T-cells to interconnect and regulate the pro-inflammatory system in psoriasis and other autoimmune conditions. It is this property of T-cells, and success of T-cell targeting therapies, that has made them the primary focus of research in the field.

Although psoriasis is recognised as primarily a T-cell driven disease, other leukocytes, such as neutrophils, have been shown to be important in psoriasis pathogenesis[723]. Neutrophils are known to aggregate in the psoriasis skin and form pustules of Kogoj and microabscesses of Munro within the epidermis[722]. Neutrophil depletion from the skin of mice with psoriasiform inflammation, has been shown to alleviate epidermal thickening[724]. Further to this, treatment of patients with psoriasis with secukinumab (an anti-IL17 neutralising monoclonal antibody) leads to an early clearance of neutrophils which temporally coincides with lesion resolution[725]. These findings, and considering the potent pro-inflammatory properties of neutrophils, suggest that neutrophils contribute to psoriasis pathogenesis. Although keratinocytes are no longer considered initiators of psoriasis pathogenesis, it is important to note that they also participate in the inflammatory process. As discussed earlier, keratinocytes can produce chemokines and pro-inflammatory cytokines, creating pro-inflammatory axes and fuelling the inflammation in psoriasis.

It is generally accepted that psoriasis pathogenesis is not a linear chain of events, it rather depends on multiple complex molecular, cellular and environmental interactions[15]. For example, the leukocyte/keratinocyte-induced pathways in psoriasis pathogenesis could act singularly, synergistically or even contradict each other. Moreover, these pathways could also be induced, stimulated or disrupted by extrinsic or other factors. All of these factors make the immunopathogenesis of psoriasis very dynamic and hard to predict. The role of different leukocytes and stromal cells in psoriasis pathogenesis is discussed below.

1.5.8.2 Dendritic cells in the immunopathogenesis of psoriasis

DCs have been long recognised as critical cells in the initiation and propagation phases of psoriasis[718,719]. As discussed earlier, multiple DC subsets exist, which have different origins, functions and characteristic. The psoriasis plaques home various DC subsets, predominantly in the dermis, which participate in plaque development; however, the relative contribution of each DC subset may vary[726,727]. The variability can be attributed to DCs ability to produce different cytokines and stimulate different responses at a given stage of plaque development. DC numbers are elevated in the involved skin of patients with psoriasis but become reduced upon treatment (e.g. anti-TNF therapy), underscoring DC importance[726,728].

The initiation phase of psoriasis plaque development is an important stage in psoriasis pathogenesis (Figure 1.4) and multiple studies have shown that pDCs actively participate during this stage[718,719]. pDC numbers have been shown to be elevated in the involved skin of patients with psoriasis when compared to healthy controls or uninvolved skin (of patients with psoriasis)[729]. pDCs participate in plaque initiation via the production of high IFN-α levels upon self-nucleic acid recognition[718,719,731]. Self-nucleic acid recognition by pDCs is usually highly regulated and preventable[730]; however, in psoriasis, genetically predisposed, stressed keratinocytes produce abundant amounts of AMPs[13,718,719,733]. It has been demonstrated that self-nucleic acids can form complexes with AMPs, such as LL-37, β-defensins, prompting recognition by TLR-7, -9 in pDCs and IFN-α production[718,719,731,733]. IFN-α is an important cytokine during the initiation phase as it can feed into the IL-17/IL-23 axis early on by inducing IL-17 expression[731,732]. This can induce upregulation of neutrophil-attracting chemokines, such as CXCL1 and CXCL8, and subsequent neutrophil recruitment[555,578,732,734]. IFN-α can also influence Th17 and Th1 polarisation, monocyte differentiation into DCs, and upregulated expression of the IL-22 receptor by keratinocytes[731,732,735,736]. Interestingly, murine studies regarding the importance of IFN-α to psoriasis pathogenesis have been contradictory. For example, psoriasis progression can be prevented by blocking the IFN-α/β receptor in the xenograft mouse model of psoriasis[731]; however, psoriasis development in the imiquimod mouse model has been shown to rely on IL-23, rather than IFN-α signalling[721]. It remains unclear if these differences are mouse model-specific or are actually related to human disease. Although anti-IFN-α human trials have

not yielded promising results, the exact role of pDC-derived cytokines in psoriasis plaque initiation remains unclear[737].

cDCs in the lesions can become activated upon stimulation with pDC-derived IFN-α and other cytokines, such as IL-6, TNF, derived from activated keratinocytes[15]. This is an important step as cDCs can produce various pro-inflammatory cytokines, such as IL-12, IL-23, IL-1, TNF[726,738]. The most pivotal cytokines being IL-12 and IL-23, as those can drive and maintain specific T-cell responses, when antigen presentation happens[739,740]. More specifically IL-12 drives Th1 responses, whereas IL-23 supports the maintenance of various IL-17-producing T-cell populations[545,726]. For example, IL-23 maintains Th17 populations and also aids the differentiation of IL-17 producing CD8+ T-cells and IL-17, -21, -22 $\gamma\delta$ T-cells[740,741]. It has been suggested that cDC dermal populations have differing capabilities when it comes to cytokine production. For example, it has been suggested that cDC1 cells in mice are better at producing IL-12 than cDC2 populations[742]. This is, however, reversed in humans and it remains unclear what the biological relevance behind this cytokine production specification is. Nevertheless, the discussed findings suggest that cDCs have the capacity to activate skin resident memory T-cells and prime naïve T-cells in draining lymph nodes. This could allow for the mounting of cDC-induced potent pro-inflammatory responses in psoriasis.

Monocyte-derived DC, referred to as inflammatory DCs (iDCs), are also present at the psoriasis plaques[743]. iDC have been shown to originate from monocytes and to be critically important for the development of psoriasis pathogenesis[744,745]. For example, targeting of Ly6C^{hi} monocytes depletes iDC levels and halts inflammation in psoriasis[745]. iDCs are not present at high levels in normal state conditions but increase rapidly during inflammation[746]. Murine studies have shown that iDC presence and recruitment to the psoriatic lesions depend on the expression of CCR2 and CCR6 by monocytes[745]. These cells are extremely potent producers of pro-inflammatory cytokines, such as IL-23, IL-1β, IL-6, and can polarise T-cells responses to a Th1, Th17 phenotype[747,748]. Studies have shown that in human psoriasis lesions iDCs are superior at driving IL-17, IL-22, TNF production by T-cells[749].

The role of LCs in psoriasis pathogenesis remains poorly understood. Studies suggest that LC numbers fluctuate throughout disease progression and that LC migration is impaired in psoriasis[738,750-752]. LCs have been shown to produce anti-inflammatory cytokines, such as IL-10, and LC depletion in mice leads to increased neutrophil infiltration[753,754]. These studies suggest that LCs might play an anti-inflammatory role in psoriasis. Contradictory studies, however, have

demonstrated that LC are IL-23 producers and therefore support T-cell responses[738]. Furthermore, LC depletion in the imiquimod mouse model of psoriasis leads to reduction of inflammation and Th17-associated cytokines[755]. Such big discrepancies in the data related to LCs could be attributed to differences in the mouse models. Nevertheless, it underscores the need for more in-depth research in the area.

1.5.8.3 T-cells in the immunopathogenesis of psoriasis

The immune pathways which participate in psoriasis initiation and onset have been characterised in greater detail and revised multiple times. Early studies demonstrated that ciclosporin, a T-cell proliferation inhibitor and now a mainstay therapeutic option, alleviates severe psoriasis[72,109]. Furthermore, it was later demonstrated that the injection of pre-psoriatic skin with CD4+ T cells was sufficient to induce psoriasis[717]. These studies underscored the role of T-cells in psoriasis and defined psoriasis as a T-cell-driven disease. Traditionally, T-cell responses used to be classified as Th1 or Th2 type of responses. The early understanding of psoriasis immunopathogenesis would postulate that Th1 cells play a dominant role in the propagation of psoriasis[756]. This belief was based on findings of elevated levels of Th1-associated cytokines, such as IFNγ and IL-2, in the plaques and periphery of patients with psoriasis[756,757]. However, the identification of a new CD4+ T-cell helper type population has challenged the Th1-driven model of psoriasis pathogenesis. Recent evidence supports the notion of a primarily IL-23/IL-17-driven pathogenesis, which centres around Th17 T-cell responses[111,112,555,734]. The role of Th17 cells in psoriasis pathogenesis will now be discussed in greater detail

1.5.8.3.1 The role of Th17 cells in the pathogenesis of psoriasis

As mentioned earlier, Th17 cells are important players in psoriasis pathogenesis. Upon provision of signal 1 (antigenic stimulation), signal 2 (co-stimulation) and signal 3 (TGF-β + IL-21) by APCs (e.g. DCs), Th17 cells develop and expand[758]. Importantly, the acquisition of Th17-specific properties is accompanied by the up-regulation and surface expression of chemokine receptors. This represents a possible migratory strategy for Th17 cells, destined to infiltrate the psoriatic lesion. For example, it has been shown that such cells up-regulate the expression of CCR6[759] which in turn allows engagement with the CCL20 chemokine, produced by IL-17/IL-22-

stimulated keratinocytes, and the consequent migration to the psoriatic skin[760]. Indeed, elevated levels of Th17 cells have been identified in the skin lesions and circulation of patients with psoriasis[761, 762].

At the site of inflammation Th17 cells will be exposed to and influenced by the local microenvironment. IL-23 is an important component of the psoriatic lesion and multiple studies have reported its overproduction in skin lesions[112,740]. Furthermore, it has been shown that cells, such as DCs and keratinocytes harbour a source of IL-23 at the inflamed psoriatic areas[568,763]. Following the i.v. introduction of the cytokine to healthy mice, psoriasis-like inflammation (e.g. epidermal hyperplasia) develops[764]. Significantly, IL-23 stimulation has been linked to the expansion of Th17 populations and augmented production of hallmark inflammatory Th17 cytokines[765,766]. Further evidence, coming from GWAS, indicates that mutations in genes, which encode proteins (e.g. IL-23R), affecting the IL-17 pathway, are associated with protection against psoriasis[208, 767]. These and further studies highlight the importance of the IL-23/IL-17-axis in autoimmunity and psoriasis (Figure 1.8).

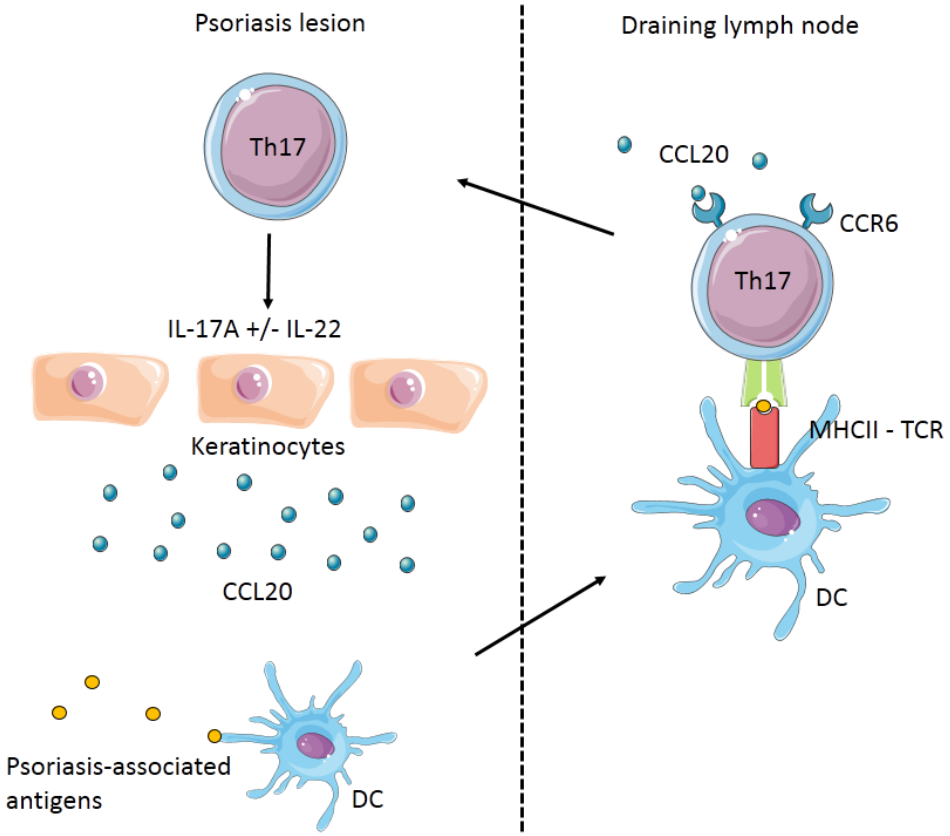


Figure 1.8| The recruitment of Th17 cells to the psoriatic skin lesion is part of a positive feedback loop.

Graphical representation of the IL-17/IL-23 pro-inflammatory loop in psoriasis. DCs, found at the psoriatic lesion, sample the microenvironment for antigens and migrate towards the draining lymph nodes. DCs have the ability to present these antigens to naïve CD4 T-cells and guide their differentiation to psoriasis-inducing Th17 cells. Furthermore, DCs promote the up-regulation of the CCL20 receptor – CCR6 – by Th17 cells, which allows them to migrate towards the psoriatic lesion. The microenvironment at the psoriatic lesion induces the production of Th17 cytokines, such as IL-17 and IL-22, which promotes the production of CCL20 by keratinocytes. This forms a positive feedback loop, as the newly produced CCL20 will further attract Th17 cells and promote the development of chronic inflammation. Original figure, visual graphics taken from SmartServier.

IL-23 stimulation of Th17 cells at the site of inflammation is a critical step in the induction of psoriasis pathology. This prompts the up-regulation and production of key inflammatory cytokines such as IL-17A, IL-17F and IL-22[734,768]. Early studies identified the presence of IL-17A in the psoriatic lesions[769]. It was not until later, however, that the production of IL-17A was, in part, attributed to Th17 cells[762,770]. It is important to note that other cells types, such as neutrophils, CD8 T cells and γδ T cells, present at the psoriatic lesion have been found to produce or store IL-17A[771-773]. Such findings suggest that Th17 cells might not be the primary source of IL-17A, at least not in all contexts. Intriguingly, the increase in IL-17A production was shown to be restricted to the psoriatic lesion, with negligible concentrations in the peripheral blood[774]. This is in contrast to other Th17-type autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis, whereby elevated levels of IL-17A have been reported in the circulation[775,776]. On the other hand, IL-22 mRNA and protein levels have been demonstrated to be elevated in both the psoriatic skin and periphery[768,777].

It is now clear that IL-17A and IL-22 have a profound effect on the surrounding stromal cells (Figure 1.5). For example, it has been demonstrated that IL-17A promotes the expression of pro-inflammatory cytokines, such as IL-6, CXCL8 and GCS-F, by epithelial, endothelial cells, and dermal fibroblasts[778]. Similar roles have been attributed to IL-22, which is known to drive the production of CXCL8 by epithelial cells[555,779]. This is important, because it represents a potential mechanism to attract neutrophils, which have once again gained prominence in the field, and further exacerbate inflammation[424]. Furthermore, the synergistic effect of IL-17A and IL-22 on keratinocytes has also been examined. The production of antimicrobial peptides (e.g. β-defensin-2, S100) and CCL20 by keratinocytes can be induced either by the synergistic action of the two key cytokines or by IL-17A alone[568,760]. This suggests the existence of a mechanism to recruit cells of both the innate and adaptive immune system. For example, as mentioned earlier, Th17 cells which bear the CCL20 receptor will be attracted to the site of inflammation. This not only creates a positive feedback loop (Fig. 2) but might be an important step in driving chronic psoriasis-associated inflammation. It is important to note, that IL-17A and IL-22 can also modulate distinct pathways within keratinocytes and thus evoke different responses. For example, in vitro exposure to IL-22 induces thickening and aberrant proliferation of keratinocytes[734]. On the other hand, IL-17A has been shown to affect the expression of adhesion molecules and filaggrin on keratinocytes, which subsequently affects the skin barriers[780]. This, once again, highlights the importance of Th17 cytokines in both skin defence and psoriasis pathogenesis.

Murine studies have been performed to examine the importance of cytokines, which either stimulate or are produced by Th17 cells in psoriasis. For example, the severity of IL-23-induced hyperplasia was reduced upon treatment with anti-IL-22 and anti-IL-17A antibodies in mice[781]. In a similar model of psoriasis, blockade of the IL-23p40 subunit was sufficient to prevent the development of skin lesions. Blockade of IL-22 had similar effects and was accompanied by the decrease of Th17-related pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-17, TNF)[782]. These and other studies serve to demonstrate that IL-17A, IL-22 and IL-23 are strong candidates for linking Th17 cells to cutaneous inflammation and the subsequent development of pathology[783]. Moreover, they underline the IL-23/IL-17-axis as a key current therapeutic target in disease management.

1.5.8.3.2 Tissue-resident T-cells in the pathogenesis of psoriasis

E-selectin is an adhesion molecule expressed by endothelial cells, which is important for T-cell migration from blood to skin during TEM. The realisation that blocking E-selectin does not positively affect psoriasis development, suggested that Trms are an important component of psoriasis pathogenesis[784]. Furthermore, the AGR129 xenotransplantation model (human non-lesional psoriatic skin transplanted onto immunodeficient mice, with no circulating lymphocytes) discussed in chapter 1.5.9.2, did not only demonstrate Trm existence[698]. This model showed that Trms can induce psoriasis plaque development in the absence of circulating lymphocytes, further underscoring the importance of Trms in psoriasis.

Very importantly, it has been demonstrated that epidermal resident T-cells that can trigger psoriasiform inflammation are present in non-lesional skin from patients with psoriasis[14]. These cells were enriched in the epidermis, CCR6+ and were able to produce IL-17 and IFNγ, which are important drivers of psoriasis immunopathogenesis. It has been suggested that such T-cell pools are generated from the interplay between genetically predisposed keratinocytes and microbes. More recent studies have tried to define the residual skin-resident T-cell populations, following plaque clearance in psoriasis. Such studies have shown that IL-17-producing CD8+ T-cell and IL-22-secreting CD4+ T-cell populations remain after plaque clearance[785]. Interestingly, CD103+CD8+ Trms were stronger IL-17 producers than CD103-CD8+ Trms, suggesting epidermal confinement[785]. Furthermore, resolved psoriasis lesions have been shown to contain psoriasis-specific IL-17-producing αβ T-cell clones[786]. These studies strongly suggest that such Trm population may be important not only during psoriasis initiation, but also relapse

of the disease. Importantly, memory T-cells are easy to activate as they do not require as strong co-stimulation and help as naive T-cells[605,609]. These cells can also generate much faster and potent immune responses, making them strongly pathogenic and harmful in autoimmune conditions, such as psoriasis[605,609]. Trms could rapidly recruit pro-inflammatory cells at the plaque via heightened chemokine production and therefore initiate, propagate or re-start plaque development. It is currently unknown exactly what role Trms play during the initiation of plaque development; what cells or molecules might activate them; what cells Trms activate early during plaque development. Examining the relationship between Trms and other leukocytes, players in psoriasis pathogenesis, could aid the identification of new treatment targets.

1.5.8.3.3 Regulatory T-cells in the pathogenesis of psoriasis

Tregs are an important component of the immune system and SALT. Tregs are critical for the maintenance of immunological homeostasis, as evidenced by individuals who lack or have dysregulated Treg responses[787]. Such patients die at a young age as they develop several autoimmune conditions due to the absence of Tregs[787]. Impairment of Treg responses has been reported in multiple autoimmune conditions, including psoriasis[788]. Such impairment could be related to defects in Treg function or resistance of effector cells to the effects of Tregs, or both. Co-culture experiments have demonstrated that IL-6 signalling could potentially impair Treg suppressor functions[789]. Treg cells and effector T-cells express high levels of IL-6R in psoriasis and IL-6 levels are also elevated in the plaques[790]. Therefore, IL-6, being a driver of T-cell proliferation, could not only impair Treg function in psoriasis but also confer resistance of effector T-cells to Treg suppression. Although it is generally accepted and has been reported that Tregs in psoriasis have impaired regulatory functions, research in the field remains highly contradictive[789]. Several research groups have reported increased peripheral blood levels of Tregs, which positively correlate with disease severity[792]. Conversely, other groups have demonstrated lower peripheral blood Treg levels, when compared to healthy controls[793]. It is believed that the variable results obtained might be due to differences in psoriasis stage and severity between patient cohorts tested. The most striking observation so far is perhaps the distorted balance created by the psoriasis microenvironment, which favours effector T-cells over Tregs[791]. Treg fate is determined by the transcription factor forkhead box protein (FOXP3); however, FOXP3 expression is finely balanced with that of RORγt, which favours Th17 fate. It has been demonstrated that psoriatic Tregs are more prone to modification of their FOXP3 expression levels[794]. Moreover, the pro-inflammatory cytokines in the psoriatic plaques have

been shown to induce RORγt expression in Tregs[794,795]. The primary driver of this is probably IL-23, as its addition drove the induction of IL-17-producing Treg populations, which were FOXP3+ RORγt+[795]. These population exhibited a gradual dimming of FOXP3 expression and concurrent RORγt induction. It is likely that this conversion is a gradual process, which is strongly dependent on the microenvironmental stimuli. Nevertheless, it holds the possibility for the development of a FOXP3- RORγt+ Th17 population from Tregs. It remains unclear if the phenotype could be reversed back to a regulatory one. Therefore, modulation of the microenvironment could represent yet another possible route for a therapeutic intervention.

1.5.8.3.4 CD8+ T-cells in the pathogenesis of psoriasis

Cytotoxic CD8+ T-cells employ various potent mechanisms to destroy target cells and have been previously implicated in psoriasis pathogenesis[772]. GWAS studies have identified the HLA-Cw6 allele of HLA-C (an MHC class I molecule) as a risk gene which positively correlates with psoriasis susceptibility[192,202]. MHC class I molecules are expressed by all keratinocytes and are involved in antigen presentation and activation of CD8+ T-cells. Therefore, implicating a role of CD8+ T-cells in psoriasis pathogenesis. Moreover, CD8+ T-cells have been shown to recognise epidermal autoantigens in psoriasis, such as melanocyte-derived antigen ADAMTS-like protein 5, in the context of HLA-Cw6[197]. However, the specific CD8+ T-cell-targeted antigen in the skin remains unknown. Considering the finding that keratinocytes in psoriasis show resistance to apoptosis, it likely that cytotoxic T-cells utilise mechanisms other than perforin/granzyme to cause disruption[796]. Indeed CD8+ T-cells in psoriasis have been shown to be prolific sources of cytokines, such as IFNγ, TNF, IL-17, IL-22[772]. This provides means to induce keratinocyte proliferation; chemokine production and recruitment of more pro-inflammatory cells. The functional significance of CD8+ T-cells has been further highlighted in the AGR mouse model, whereby CD8+ T-cells have been demonstrated to accumulate in the epidermis[698]. This accumulation has been associated with increased cytokine production and keratinocyte proliferation in the plaque. Importantly, plaque development halts upon anti-CD8 antibody treatment or blockade of CD8+ T-cells migration to the epidermis (by targeting α1β1 integrin)[797,798]. Therefore, it could be hypothesised that the early arrival of CD8+ T-cells to the epidermis precedes the activation of pathogenic Th17 responses. It has been suggested that CD8+ T-cell responses in psoriasis might be modulated by IFN-α. IFN-α induces cross-presentation of antigens, and therefore CD8+ T-cell priming, and enhances the expression of

MHC class I molecules (e.g. HLA-Cw6)[799]. Mouse studies have also tried to evaluate the impact of psoriasis-specific signalling pathways, such as the RAS signalling pathway, on the polarisation of skin infiltrating T-cells[800]. One such study showed that when RAS is constitutively expressed in mouse epidermis, a psoriasis-like phenotype develops, including keratinocyte hyperproliferation, acanthosis, neutrophil and T-cell recruitment. This phenotype was saved if the mice were immunodeficient or if CD8+ T-cell were depleted. Interestingly, immunodeficient mice with constitutive RAS signalling would develop a psoriasis-like phenotype upon reconstitution with purified CD8+ T-cells. It must be noted that the RAS-signalling pathway is not the only overactive pathway in the psoriasis lesions. Therefore, for definitive conclusions to be drawn, the converging effects of multiple pathways in directing the T-cells responses must be considered. Nevertheless, this study underscores the importance of CD8+ T-cells in psoriasis pathogenesis. It is also worth mentioning, that a lot of the CD8+ T-cells in the skin are of a memory resident phenotype and are therefore important players in immune protection[785]. Targeting autoreactive CD8+ T-cells could be a challenge, as they share features and functions with their counterparts, which participate in protection against pathogens.

1.5.8.3.5 γδ T-cells in the pathogenesis of psoriasis

Th17 are typically recognised as the prototypical source of IL-17 in the immune system. This notion has recently been challenged with the finding that γδ T-cells can produce large amount of the cytokine (i.e. IL-17)[741]. Elevated γδ T-cell have been identified in the skin of both murine models of psoriasis and patients with psoriasis[741,755,771]. More than a two-fold increase in dermal γδ T-cell numbers has been noted in the latter. Furthermore, a unique population of CLA-expressing, skin-homing γδ T-cells has been identified recently and have been reported to be elevated in patients with psoriasis[801]. Importantly, γδ T-cells express high levels of IL-23R, CCR6 and RORγt[802,803]. IL-23R expression is a characteristic of both human and murine γδ T-cells and allows the cells to rapidly respond to stimuli and produce IL-17[802,803]. Furthermore, it has been shown that IL-23 stimulates γδ T-cell expansion. CCR6 expression is also important in psoriasis as its ligand, CCL20, is upregulated in plaques, prompting γδ T-cells recruitment[802]. Therefore, γδ T-cells are equipped with receptors and machinery to supplement and amplify the Th17 responses, detrimental to psoriasis pathogenesis. Importantly, CCR6, IL-23R expression and IL-17 production could allow γδ T-cells to participate in the IL-23/IL-17 pro-inflammatory axis in

the skin plaques of patients with psoriasis. The implications of this molecular expression profile and γδ T-cells functions in psoriasis has been highlighted by murine studies[741]. For example, mice deficient in TCR δ exhibit reduced skin hyperplasia, pathology and IL-23-induced skin inflammation. Importantly, γδ T-cells can produce IL-17 in response to other stimuli, such as IL-1 and danger signals[802]. IL-17 production is particularly important in psoriasis pathogenesis, as it drives the production of chemokines and subsequent recruitment of pro-inflammatory cells, such as neutrophils and T-cells. The understanding of the role of γδ T-cells in the pathogenesis of psoriasis in humans is very limited. Nevertheless, the ability of γδ T-cells to participate in important pathogenies axes, suggests that they can affect a series of critical cellular interactions in the cascade.

1.5.8.4 The role of keratinocytes in the pathogenesis of psoriasis

Keratinocytes have been termed the main responder cells in psoriasis (Figure 1.9). This is because keratinocytes integrate molecular signals from various cellular sources, participating in psoriasis pathogenesis, and elicit a response[708,709]. Keratinocytes express various receptors, including TLRs and can therefore participate in immune surveillance; however, this also allows them to detect other signals, such as LL-37/self-nucleic acid complexes, and enhance the pathogenic immune responses in psoriasis[708,709]. As discussed earlier, stressed keratinocytes in psoriasis release large amounts of AMPs, such as LL-37, β-defensins, S100, and self-DNA/self-RNA, which can form complexes[711,712,714]. AMP/Self-nucleic acid complexes do not only activate plasmacytoid dendritic cells, they can also activate TLR-8-expressing keratinocytes to produce IFN-α[718,804]. Importantly, LL-37 alone can stimulate keratinocytes to produce various pro-inflammatory molecules, including IL-36, CXCL1, CXCL8, CXCL10, CCL20. This positive feedback loop (KC-LL-37-KC) can result in the recruitment and activation of more pro-inflammatory cells, such as neutrophils and T-cells. Furthermore, studies suggest that in the early stages of plaque development, keratinocytes produce chemerin, which is known to recruits pDCs. This could be a possible mechanism important during the very early stages of plaque initiation. Stressed keratinocytes have also been found to produce cytokines, such as IL-6, TNF, IFN-α, and IL-1, therefore supporting different aspects of the immune response. IL-1 production can induce monocyte and neutrophil recruitment and activation, aiding innate immune responses. IL-6 has been implicated in the differentiation of Th17 adaptive immune responses. The initial stages of psoriasis development are therefore marked by the response of keratinocytes to different

stimuli. These events lead to the production of mediators, which aid the initiation and propagation of the pathologic immune responses in psoriasis.

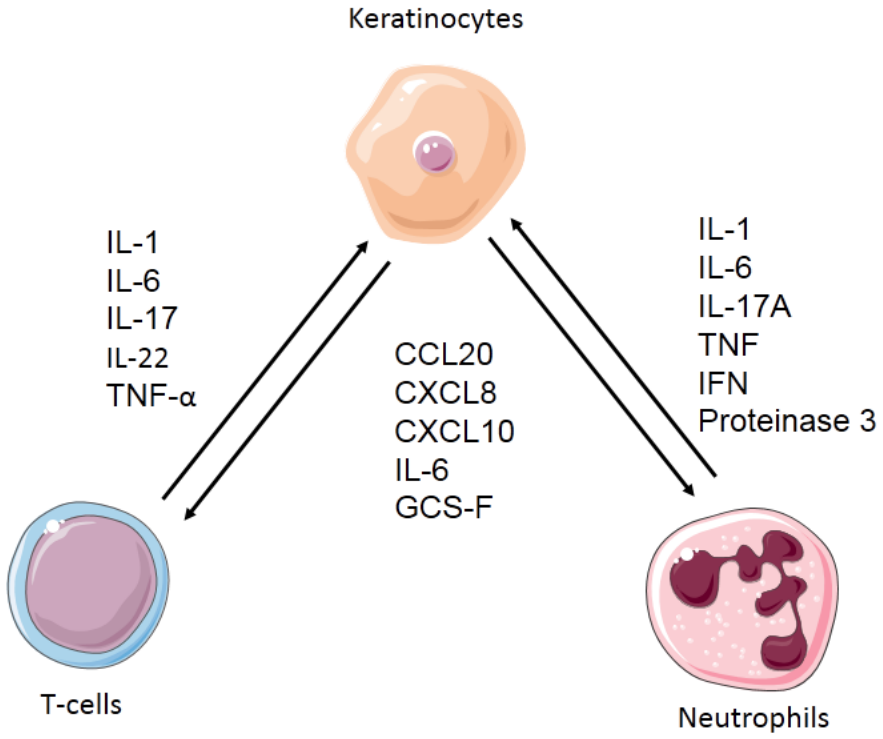


Figure 1.9| Keratinocytes are the main responders in psoriasis

A graphical representation of the complex interactions that exist between T-cells-Keratinocytes-Neutrophils in psoriasis. Both T-cells and neutrophils are early arrivals at the psoriasis lesions and are known to establish multiple interactions with keratinocytes. These interactions are; however, incompletely understood and many questions remain unanswered. For example, it is not clear which leukocyte type arrives first at the site of inflammation and to what extent the early arrivals can potentiate keratinocyte-induced leukocyte recruitment. Original figure, visual graphics taken from SmartServier.

In the later stages of psoriasis plaque development keratinocytes retain many of their characteristics that were key to the development of the initial stages; however, the overwhelming influx of leukocytes results in the increase of pro-inflammatory signals keratinocytes need to process. Therefore, keratinocytes induce a dramatic amplification of the immune responses, which results in the formation of pro-inflammatory axes. The formation of various pro-inflammatory axes is a fundamental moment during plaque development, as it allows for the inflammatory process to become self-sustained. Generally, different cytokines will prompt different responses by keratinocytes, but these can overlap, feed into or contradict each other. For example, both IFNγ and IL-17 levels are highly elevated at the lesion sites and are produced by Th1 and Th17 cells, respectively. The receptors for both are expressed on keratinocytes and signalling through them results in the production of various chemokines. Such chemokines include CCL2, CCL5, CCL20, CXCL1, CXCL3, CXCL5, CXCL8 and induce the recruitment of various leukocytes, such as T-cells, neutrophils, monocytes[805-807]. These leukocytes can further fuel this process. In addition to chemokines, AMPs production by keratinocytes also becomes elevated, increasing the chances of autoantigen assembly. AMPs can also act as chemoattractants for DCs, neutrophils, T-cells[808,809]. TNF is another cytokine, overexpressed at the lesions, which stimulates the production of aforementioned chemokines by keratinocytes[295]. Furthermore, TNF induces the expression of adhesion molecules, such as ICAM-1, by keratinocytes and increases chances of leukocyte recruitment[810]. TNF and IL-1 also upregulate vascular endothelial growth factor expression by keratinocytes and therefore stimulate angiogenesis and leukocyte recruitment[811]. TNF can also induce production of IL-6 and IL-1 by keratinocytes and stimulate Th17 differentiation and subsequent production of IL-17 and IL-22[295]. IL-22 is another important player in this process and its receptor is expressed by keratinocytes. IL-22 not only drives chemokine and AMP production but also potentiates keratinocyte proliferation and epidermal acanthosis[734,735].

Therefore, keratinocytes are equipped with various receptors which allow them to integrate many signals simultaneously. Although this is important during the initiation of psoriasis plaque development, it becomes even more prominent during the later stages of the disease. Keratinocytes act to amplify all of those signals which ultimately leads to the formation of multiple positive pro-inflammatory loops and permanent dysregulation of skin homeostasis.

1.5.8.5 The role of neutrophils in the pathogenesis of psoriasis

Neutrophils infiltrate the dermis early in psoriasis and subsequently aggregate in the epidermis, often forming Munro's microabsseces[722]. The increase in neutrophil recruitment could possibly be

explained by the rapid increase of neutrophil chemoattractant levels at the plaques. It has been shown that neutrophil migration to the psoriasis plaques is particularly dependent on the upregulation of specific adhesion molecules, such as Thy-1[812]. It is, however, unclear if a specific set of adhesion molecules mediates neutrophil recruitment to the skin in psoriasis. Furthermore, neutrophil microabscess formation has been shown be, at least partially, IL-6-driven[813]. This was demonstrated in a murine model of psoriasis, driven by IL-17-induced inflammation. Therefore, IL-6 signalling may function downstream of IL-17 to mediate neutrophil clustering. Importantly, neutrophils express receptors for both IL-17-induced chemokines (e.g. CXCL8) and IL-6, suggesting that this could be indeed an important pathway in microabscess formation[406,410,813]. Despite their presence being long recognised for some time, their functional role has remained poorly understood. Neutrophils store and produce a plethora of potent pro-inflammatory mediators, such as chemokines (e.g. CXCL8), antimicrobial peptides (LL-37), and cytokines (e.g. IL-17)[723]. The release of such mediators can drive inflammation via the recruitment of leukocytes and stimulation of neighbouring cells. Importantly, neutrophils can also release self-nucleic acids, during a process known as NETosis, thus increasing the assembly of autoimmune complexes[814,815]. The psoriasis plaque microenvironment is rich in various stimuli (e.g. CXCL8, TNF, IL-1), which stimulate and favour NETosis activation by neutrophils[430,431]. It has been shown that patients with psoriasis have increased peripheral blood levels of NETotic cells, when compared to healthy controls[816]. Further to this, the increase in NETotic cells was positively correlated to disease severity, demonstrating the importance of this phenomenon in psoriasis[816]. Importantly, the aforementioned NETosis stimuli can also affect neutrophil respiratory burst. Therefore, neutrophils present at the plaque are more prone to experience a respiratory burst and have been shown to have an enhanced myeloperoxidase (MPO) and NADPH oxidase activity[817,819]. These enzymes contribute to the respiratory burst and reactive oxygen species (ROS) formation. Ineffective ROS clearance causes oxidative stress and can lead to extensive tissue and cellular damage, enhanced keratinocyte proliferation, induction of angiogenesis[820,823]. The accumulation of oxidative stress can also trigger pDC activation and stimulate antigen presentation, therefore influencing adaptive immune responses[819]. Furthermore, MPO can drive neutrophil degranulation[821]. Neutrophil degranulation is a common process, which takes place in psoriasis plaques, and involves the release of various pro-inflammatory molecules and proteases (e.g. MPO, proteinase 3, neutrophil elastase)[821,822]. The release of such molecules can further aggravate the inflammatory response and damage the integrity of the skin (e.g. proteinase 3 aids LL-37 release[824]). It is important to mention that NETosis and degranulation also results in the release of cytokines, chemokines, AMPs[423,424,427,825]. IL-17 is an important cytokine in the inflammatory cascade which drives psoriasis development. The role of neutrophils in the IL-17

cascade has gained a great deal of appreciation in the last decade. IL-17 inhibition via secukinumab in psoriasis patients, leads to a marked reduction of skin neutrophils and subsequently limited cross-talk with keratinocytes[725]; yet the full functional significance of this remains unexplored. The abolished epidermal microabscess and reduced T-cell activity, suggest a possible role of neutrophils in the IL-17/IL-23 axis and localisation within skin. Neutrophils have been shown to express low levels of IL-17 mRNA, suggesting they are not prolific active producers of the cytokine[826]; however, they are able to store IL-17 in their granules/cytoplasm and release it upon degranulation[825]. This could allow neutrophils to feed into the IL-17/IL-23 axis and likely contribute to T-cell and further neutrophil recruitment. It remains unclear whether neutrophils have an effect on resident Th17 cells or if resident T-cells become activated early in psoriasis and induce neutrophil recruitment. It is also unknown what drives neutrophil accumulation within the epidermis.

1.5.8.6 The role of chemokines in psoriasis pathogenesis

The recruitment of pro-inflammatory cells and their positioning within tissues is highly dependent on chemokines. Very importantly, the interactions between different leukocytes in the psoriasis lesion are orchestrated by the chemokine/chemokine receptor system. One such pivotal interaction, which is largely chemokine-dependent, is between neutrophils, T-cells and keratinocytes[568,825,827]. As discussed in this thesis, both neutrophils and T-cells are critical to induction and development of the psoriasis plaques. The early infiltration of neutrophils in psoriasis and their strong inflammatory profile, suggests that they might be responsible for the keratinocyte-dependent recruitment and activation of distant and resident T-cells[25,722]. It is also possible that the early neutrophilic infiltration might be a result of T-cell-dependent production of keratinocyte-derived chemokines[568,691,828]. This issue has never been addressed before. Multiple neutrophil and T-cell attracting chemokines have been found to be elevated in the lesions of psoriasis patients, but their functional relevance remain largely unexplored (Table 1.2).

The positioning of the T-cells within the psoriatic lesion is of particular importance. As discussed earlier, the skin contains a large population of TRMs[691]. These cells are more prone to activation and are potent cytokine producers[605,829]. The AGR129 xenotransplantation model (human non-lesional psoriatic skin transplanted onto immunodeficient mice, with no circulating lymphocytes) demonstrated that the migration of TRMs into the epidermis is sufficient for plaque

development[698,828]. Further to this, CD8+ T cells and neutrophils are a rich source of IL-17 and aggregate within the epidermis[772,825]. Blocking the dermal-epidermal transition of CD8+ T-cells in the AGR129 model, limits disease development9. This serves as evidence for the correlation between disease progression, accumulation of inflammatory cells within the epidermis and the importance of leukocyte migration in psoriasis. Although these experiments demonstrate the importance of leukocyte positioning within skin lesions using a xenotransplantation murine model, anti-psoriatic therapies that specifically target cell migration and tissue positioning, both critical for inflammation, are currently lacking.

CCL20 and CXCL8 are examples, considered critical for the induction and maintenance of the pro-inflammatory feedback loops in psoriasis[760,830]. As discussed earlier, CCL20 levels in the psoriatic lesions have been reported to be highly elevated, when compared to healthy skin. It has been suggested that this is a result of the highly inflammatory cytokine milieu at the lesions. Importantly, CCL20 binds to only one receptor – CCR6 – and can therefore recruit specific CCR6-expressing leukocytes[360]. Further to this, CCL20 has been found to be elevated in dermal endothelial cells and could therefore participate in the arrest of CCR6+ cells from the blood stream[831]. Indeed, CCR6 has been shown to mediate adhesion of T-cells under conditions of shear stress[832]. CCR6+ T-cells have been shown to be elevated in the periphery and lesions of patients with psoriasis[759]. The importance of the CCR6/CCL20 system has been further highlighted by animal studies. For example, murine studies have shown that the induction of a psoriasis phenotype is abrogated in CCR6 deficient mice[833]. It should also be taken into account that CCR6 defines and is highly expressed by Th17 cells, the main T-cell drivers of psoriasis pathogenesis[337,340,447]. Apart of chemokines, β-defensins, also upregulated in the lesions, also engage in interactions with CCR6 and can therefore act in unison with CCL20 to attract leukocytes[834]. It remains unknown what the initial drivers of CCL20 production are and CCL20-orchestrated sequence of events in psoriasis immunopathogenesis. Although we have a better understanding of the CCL20/CCR6 system, the recruitment of T cells in the context of psoriasis remains poorly defined. The pattern of chemokine receptor expression on effector T cells is complex and the factors controlling the expression of those receptors are also unclear. Although some chemokine/receptor pairs are known to be important for cell migration to specific tissues, others do not show such associations. Elevated levels of CCL2, CCL5, CCL17, CCL18, CCL27, CXCL10, CXCL16 and CX3CL1 (effector T cells bear adjacent receptors) have been previously reported in psoriasis lesions (Table 1.2). It is not known whether the aforementioned chemokines attract T cells in the context of psoriasis and what cellular interactions lead to their elevated production.

Similarly to CCL20, levels of CXCL8 (a neutrophil attractant) have been demonstrated to be elevated in psoriasis lesions[2]. CXCL8 competes with chemokines, such as CXCL1 and CXCL2, to binds CXCR1 or CXCR2. The neutralisation of CXCL8, does not abrogate neutrophil recruitment and the inflammatory response, suggesting redundancy in the chemokine network (CXCL1 and CXCL2 bind the CXCL8 receptor)[835]. The role of other neutrophil attractants is even less well understood. For example, neutrophils upregulate CCR2 expression during times of inflammation[835], but it is unclear whether CCL2 (a CCR2 ligand) accounts for cell recruitment in psoriasis. Furthermore, CXCL16 has been shown to be upregulated at the psoriasis lesions and to induce neutrophil recruitment *ex vivo*; however, CXCL16 only induced neutrophil recruitment when at extremely high concentrations or in conjugation with CXCL8[837]. Such data underscores the need for a better understanding of how chemokines might work together to recruit cells to the lesions and facilitate their intra-lesional movement. For example, it is unclear if the chemokine receptor combination needed for lesion homing is similar to the one required for dermal-epidermal transition. Importantly, these are not isolated examples, as our understanding of the role of chemokines in psoriasis is very much deficient.

Critically, chemokines control leukocyte migration and provide direction for migration. Psoriasis is a systemic condition associated with elevated levels of many different pro-inflammatory factors, many of which discussed in this thesis. As mentioned previously, such pro-inflammatory mediators markedly increase chemokine production. It is, however, unknown if systemic inflammation also influences the ability of leukocytes to respond to chemokine signals. It has never been demonstrated if increased lesional leukocyte numbers could be a result of intrinsic leukocyte changes, such as elevated migratory potential. If psoriasiform inflammation is marked by an increase in the migratory potential of leukocytes, understanding such characteristic changes could help uncover novel treatment targets. It is also unknown if current psoriasis treatments affect leukocyte migratory potential and responsiveness to particular chemokines. Even less clear is the sequence of migratory events and how they might be modulated by the pro-inflammatory environment and potential changes in leukocyte migratory potential. For example, if the outcome of T cell-stromal cell interactions is neutrophil recruitment, or vice versa. A critical step in psoriasis pathogenesis is epidermal infiltration by immune cells; however, there is no clear evidence on what chemokine/receptor couple(s) orchestrate these events. Deciphering these cellular interactions will give us a better insight into disease initiation and progression, potentially unmasking novel strategies for future therapy development.

1.6 Immunological parallels between psoriasis and psoriasis-like and psoriasis-associated conditions

1.6.1 Psoriatic arthritis

Psoriasis is often (in approximately 30% of the patients) further complicated by the development of psoriatic arthritis (PsA), which can cause destructive arthritis and, in some cases, permanent loss of function[5]. The combination of these conditions can significantly reduce patients' quality of life and is associated with the development of other diseases (e.g. diabetes, atherosclerosis)[4].

Although the exact cause and immunopathogenesis of PsA is not entirely clear, it is known that it shares many similarities with psoriasis, with similar (albeit not identical) treatment responses. For example, Th17 cell numbers have been found to be elevated in the circulation, synovial tissue and synovial fluid of patients[22,23]. This has been linked to an increase in IL-23 production and responsiveness in the synovium. The binding of IL-23 to its cognate receptor on Th17 cells prompts the production of cytokines, such as IL-17A and IL-22. Importantly, the fibroblast-like synoviocytes have been shown to have an elevated IL-17R expression, leading to the enhanced production of detrimental pro-inflammatory mediators. For example, *in vitro* simulation induces the heightened production of IL-6 and CXCL8, which could potentially serve to exacerbate the acute phase of inflammation and recruit neutrophils to the site[23]. Therefore, the IL-17/IL-23 pro-inflammatory loop is a striking similarity between psoriasis and psoriatic arthritis. Furthermore, much like psoriasis, very little is known about the sequence of migratory events and the role of chemokines in the orchestration of the pathology. For example, it is still to be elucidated whether the Th17 cells found in the inflamed synovium derive from the skin lesions or elsewhere; what chemokines and signals might drive the translocation and recruitment of the T-cells; to what extent psoriasis and PsA are inter-linked. Therefore, the better understanding of leukocyte migration and chemokine responsiveness in the context of psoriasis could elucidate the same problems in conditions, such as PsA.

1.6.2 Neutrophilic dermatoses

Neutrophilic dermatoses (NDs) are a group of cutaneous conditions characterised by the accumulation of sterile, neutrophilic infiltrates in the skin. The clinical manifestation of the conditions may vary and the cutaneous lesions may appear as pustules, nodules, ulcers, plaques, abscesses. NDs include conditions, such as Sweet's syndrome, hidradenitis suppurativa (HS), pyoderma gangrenosum (PG) and Behcet's disease but their immunopathogenesis remain very poorly understood. It has been suggested that NDs should be categorised as autoinflammatory conditions. This is because an increase in pro-inflammatory cytokines (e.g. IL-1β, IL-17, TNF) in the absence of infection, autoreactive T-cells and autoantibodies has been noted. Importantly, the levels of chemokines, such as CXCL8 and CXCL1/2/3, in patients with PG has been reported to be elevated. This suggests that leukocyte migration plays an important role in the development of ND pathogenesis and that its dysregulation may prompt disease progression. The perception of NDs as autoinflammatory type of conditions was further highlighted by the success of IL-1β blocking therapies in the treatment of some NDs. Although dysregulated neutrophil function and chemokine production has been reported, these have not been addressed by current treatment strategies before. Evidence on what triggers the immune dysregulation and what cells participate in lesion development is very limited. Although T cell imbalance, in favour of Th17 cells, has been described, it is unknown what cells and chemokines may drive this93. Furthermore, it has been reported that the immunopathogenesis in HS might be driven by impaired Notch signalling. This might induce the increased production of IL-23 by DCs and subsequent Th17 polarisation. Therefore, evidence surrounding NDs can be contradictory and disease specific. Nevertheless, the abnormal neutrophilic behaviour and presence of pro-inflammatory cytokines, which can stimulate chemokine production, is similar to psoriasis. It remains unclear, however, what the sequence of event in disease initiation and progression is and what molecules and cells might be driving it. Therefore, drawing parallels between NDs and other, better understood, skin conditions may aid the identification of new therapeutic targets for the treatment of NDs.

1.7 Mouse models of psoriasiform inflammation

Psoriasis is a disease which almost exclusively affects humans. The lack of animal models has made *in vivo* research of psoriasis pathogenesis challenging. However, the development of

mouse models of psoriasiform inflammation, over the past two decades, has made pre-clinical animal research possible[838]. It is important to underline that human psoriasis is still different to the *psoriasiform* inflammation induced in mice, making data interpretation difficult in certain scenarios. In order for a mouse model to be considered successful it should replicate the key histological features of human psoriasis. Such features being parakeratosis (retention of nuclei in the S. corneum), hyperplasia, acanthosis (thickening of the skin), vascular proliferation, elongated rete ridges, a prominent leukocyte infiltrate, skewed T-cell immunity[839]. Mouse models of psoriasiform inflammation are met with certain limitations. For example, mouse models do not fully reproduce the aforementioned characteristics. This could be explained by the fundamental differences between human and mouse skin, immune system, genetics, habitable environment. For example, mouse epidermis (only 2-3 keratinocyte layers) and dermis are less thick than human and hair follicles are much more prominent[838-840]. Furthermore, the short inter-follicular regions in mouse skin do not contain rete ridges, whereas this is not the same in human skin. Immune system differences regard leukocyte populations (e.g. dendritic cells). The main genetic difference is that human psoriasis is a result of complex multi-gene interactions, rather than the manipulation of a single gene target[838-840]. Although no mouse model can fully replicate human psoriasis, mouse models have helped us better understand the disease and given us clinically-relevant results. Several mouse models of psoriasiform inflammation have been developed; however, for the purpose of this thesis only the imiquimod and xenograft animal models of psoriasiform inflammation shall be further discussed.

1.7.1 The Imiquimod mouse model

Imiquimod was originally developed as an antiviral and is used for the treatment of genital warts, actinic keratoses, and basal cell carcinomas. It is suitable for topical administration due to its small molecular size and high hydrophobicity and is marketed as Aldara™ cream (5% Imiquimod)[841]. Imiquimod efficacy has been attributed to its ability to induce local activation of the immune responses[721]. Its repeated application to mouse skin has been shown to induce psoriasiform inflammation. The imiquimod mouse model is usually induced on the back skin or ears of C57BL/6 mice. Imiquimod activates the immune response through TLR-dependent and independent pathways in a phased manner[721]. TLRs which become activated by imiquimod are TLR7, TLR8 in humans and TLR7 in mice[842,843]. Murine cells which express TLR7 are monocytes, macrophages, pDCs and respond to imiquimod by producing pro-inflammatory cytokines. It has been shown that this happens via the MyD88-dependent activation of the NF-kB pathway[842,843].

Therefore, imiquimod-induced activation of psoriasiform inflammation is comparable to the pDC-dependent induction pathway of human psoriasis. In addition to TLR activation, imiquimod has also been shown to activate the inflammasome complex, presumably by keratinocytes[844]. Importantly, the imiquimod carrier also participates in the induction of inflammation[844]. Aldara vehicle can activate the inflammasome in keratinocytes and this has been shown as an important event during the early phase of the Aldara mouse model. However, the fully pledged imiquimod mouse model of psoriasiform inflammation remains largely dependent on signalling through MyD88. Mice deficient in MyD88 fail to develop the phenotype[845].

The first 24h after Aldara application are marked by the production of IFN-α, IL-1, IL-6, and later IL-23[844,845]; recruitment of neutrophils and mast cells; abscess formation; keratinocyte proliferation; epidermal cell death[844,846]. Interestingly, the induction of these cytokines in the early phase is MyD88-, but not TLR7-dependent[845]. This suggest that the MyD88 pathway becomes activated as a result of the ligation of different to TLR7 receptors. It remains unclear what that receptor might be. It has been suggested that mast cells are important during the early induction phase. Mice deficient in mast cells show a reduced early inflammatory response following Aldara application[846]. The role of the cytokines, which characterise the first 24h after Aldara application, in the development of psoriasiform inflammation has been investigated. For example, IFN-α signalling has been found to be dispensable for the development of the psoriasiform phenotype in this model[844,845]. Similarly, IL-1R deficient mice do not show changes in skin thickening, albeit diminished neutrophil microabscess formation[847,848]. However, other IL-1 family members, such as IL-36, have been shown to be critical for the development of psoriasiform inflammation in the imiquimod mouse model[848]. The production of IL-23 at the end of the early phase has been shown to be dependent on conventional DC activation via the MyD88 pathway[721,845]. IL-23 production also marks the late stages of the imiquimod mouse model of psoriasiform inflammation.

In addition to IL-23 production, the late phase is characterised by a new wave of neutrophil recruitment and $\gamma\delta$ T-cell accumulation after day 4, peaking at day 7[848]. Studies suggest that neutrophil recruitment in this model is CXCR2-dependent[849]. IL-23 has been shown to be critical for the induction of the late phase and the Imiquimod mouse model in general. IL-23R deficient mice are protected from disease. Although IL-23 is induced at 24h post application, its production peaks at 48-72h following multiple Aldara cream applications[721,845]. IL-23 induces the production of IL-17 family of cytokines and IL-22, which peak 24h after the IL-23 peak[580]. IL-17A, IL-17F, IL-22 deficient mice are only partially protected against disease, suggesting these cytokines have a synergistic action[771,848,851]. Interestingly, IL-17F has been found to be the

most important of the IL-17 family of cytokines for the induction of the imiquimod mouse model of psoriasiform inflammation[851]. IL-17 and IL-22 induce chemokine production and lead to the recruitment of more pro-inflammatory cells[721,768,771]. IL-17 action has been previously associated with neutrophil recruitment, whereas IL-22 can drive keratinocyte hyperplasia[721,768,771]. The principal producers of IL-17 and IL-22 in this mouse model are γδ T-cells[771,850]. They respond to the IL-23 signals by producing abundant levels of the pro-inflammatory cytokines and drive the inflammatory response[771]. It has been suggested that IL-22 is also produced by innate lymphoid cells and that IL-17 can be produced by CD4+ T-cells in the absence of γδ T-cells[848,851]. These findings suggest that the imiquimod mouse model of psoriasiform inflammation is dependent on the IL-23/IL-17 pro-inflammatory axis.

1.7.2 Xenotransplantation mouse model

The xenotransplantation mouse model of psoriasis is one of the most useful animal models of psoriasis to be developed. It involves the transplantation of uninvolved or involved skin of patients with psoriasis onto immunodeficient mouse background (athymic nude mice; AGR129 mice)[698]. In some scenarios plaque-supporting leukocytes need to be supplied to the graft via injection[852]. This model of psoriasis is very costly and requires acquisition of human psoriatic skin, making it less convenient than, for example, the imiquimod mouse model.

An example of this model is when human skin is transplanted onto severe combined immunodeficiency (SCID) mouse background[852]. SCID mice are devoid of T and B lymphocytes and therefore the graft does not become rejected. This model also requires the intradermal injection of autologous CD4+ T-cells, activated by IL-2 and bacterial-derived superantigen (SEB/SEC2). However, these mice still have mature natural killer cells and neutrophils, which is a major limitation. Mature natural killer cells can target the injected autologous cells and destroy them, ultimately leading to reduction of psoriasis-associated inflammation. Due to this limitation, the SCID xenotransplantation model can only be used for studying the early events of the inflammatory cascade in psoriasis.

Another xenotransplantation model of psoriasis involves the transplantation of skin onto AGR129 mouse background[698,731]. AGR129 mice lack receptors for IFN type I and II, which deprives them of T and B lymphocytes and functional natural killer cell responses. This not only allows for the graft not to be rejected but also negates the need for intradermal injection of

autologous lymphocytes to maintain the graft phenotype. In this model of psoriasis, the resident leukocytes from the transplanted skin become activated as a result of the transplantation procedure (the so-called Koebner phenomenon). The development of this model relies on the local activation of resident memory T-cells within the skin graft, as no T-cells have been found in the recipient's lymphoid organs[698,731]. A psoriasis lesion takes 35 days to develop in this mouse model of psoriasis.

The xenotransplantation models are extensively used for the assessment of response to new therapies and have given us critical insight into psoriasis development. The majority of these findings are discussed in the thesis, as they are tightly related to T-cells role in psoriasis and migration.

1.8 Thesis overview and aims

Psoriasis is a chronic, immune mediated disease, which affects 1-3% of the worldwide population[1]. Psoriasis has a profound impact on those affected in terms of morbidity and mortality. The most common clinical psoriasis subtype is psoriasis vulgaris, which is also the topic of this thesis. Psoriasis vulgaris is characterised by the development of red skin plaques covered with silvery scale. Despite its characteristic skin lesions, there is increasing recognition that psoriasis is a systemic disorder associated with high levels of circulating pro-inflammatory cytokines[2]. Furthermore, there is an excess incidence of significant co-morbidities, including cardiac and metabolic disorders, and psoriatic arthritis[3,4,5] Psoriasis is a complex multi-factorial disease, which is a result of environmental, genetic and immune system interactions. Linkage, association, and GWAS have identified and mapped psoriasis susceptibility loci[193,201]. Known environmental triggers of psoriasis are certain medications (e.g. imiquimod)[210]; Koebner phenomenon-triggering interventions (e.g. tattoo, surgery and other forms of physical trauma) and Streptococcal infections[32,720]. Due to the reasons outlines above, treatment of psoriasis is often complex, difficult and costly.

Psoriasis is characterised by the infiltration of inflammatory cells into the dermal and epidermal skin layers, hyperproliferation and abnormal differentiation of keratinocytes[12,13]. Psoriasis is considered a T-cell-driven disease[14]. Recent findings have identified Th17 cells and the IL-23/IL-17 axis as central to the development of pathology[111,112,783]. Th17 cells drive the immune responses in psoriasis by co-operating and interacting with various other leukocytes, such as neutrophils, dendritic cells, macrophages. These interactions result in the generation of multiple

pro-inflammatory cytokines and signals integrated and multiplied by the principal responder cells in the skin – keratinocytes. Examples of psoriasis-associated elevated pro-inflammatory molecules include IL-17, IL-23, TNF-a, IL-6, CXCL8, CCL20, CXCL16, CXCL10[2]. The significance of many of those has been confirmed by success of cytokine-targeting drugs for the treatment of psoriasis. However, the majority of the approved agents for the treatment of psoriasis target immune cell function non-specifically, or target selected pro-inflammatory cytokines (e.g. TNF, IL-23, IL-17). Although these have proven effective, new evidence suggests that the targeting of different immune pathways, such as immune cell migration, might improve treatment specificity[798,853].

Chemokines are the principal orchestrators of cell migration. The leukocyte interactions in psoriasis are dependent on cell migration and therefore chemokines. Leukocyte migration is a key early event in psoriasis, and it has become clear that leucocyte positioning within the skin is important for the development of the psoriatic plaque[798]. This statement is true for multiple events of the psoriatic cascade, such as dermal-epidermal crossing of leukocytes, but also the chronic disease stages. Some of the most efficacious biologic agents available appear to profoundly impact leukocyte positioning in skin[725]. However, an effective leucocyte chemotaxis targeting agent is yet to be developed. This might stem from the fact that our understanding of the chemokine-orchestrated events in psoriasis is very much deficient. For example, investigation of the migratory capacity and poise of leukocytes to migrate to the skin in psoriasis is lacking. Studying the migratory potential of leukocytes, as a marker of inflammation, in psoriasis, could allow us to better understand leukocyte interactions and drug treatment outcomes.

As discussed earlier, multiple chemokines have been found to be elevated at the psoriasis lesions[2]. However, the ability of psoriasis-associated chemokine/receptor couple(s) to orchestrate leukocyte recruitment in the context of psoriasis remains unclear. All of this is further complicated by the dynamic nature and expression of chemokine receptors. Targeting chemokines directly has historically been challenging due to the nature of chemokine biology and due to pharmacological hurdles. Leukocytes at the lesions are exposed to various signals, which might affect the expression of chemokine receptors and subsequently their migratory pattern. Such changes may even account for the critically important dermal-epidermal transition of leukocytes; however, the factors and cellular interactions controlling the expression of chemokines and their receptors are also unclear. Studying the ability of psoriasis-associated chemokines to attract leukocytes could allow us to identify new therapeutic targets. Furthermore,

it could be utilised as means to select leukocytes, likely to migrate to the lesions, for stromal-leukocyte interaction studies.

Early psoriasis lesion recruits include T-cells and neutrophils and both cell types are known to induce production of T-cell and neutrophil-attracting factors by keratinocytes. However, it remains unknown what cellular interactions trigger the leukocyte recruitment cascade at the very early stages of disease development. For example, it is not clear if the outcome of T cell-stromal cell interactions is neutrophil recruitment, or vice versa. Studying these cellular interactions will give us a better insight into disease initiation and progression, potentially unmasking novel strategies for future therapy development.

As discussed earlier, some of the in-use treatment options for psoriasis appear to profoundly impact leukocyte positioning in the skin. However, their direct impact on leukocytes' ability to migrate has never been addressed. It is worth mentioning that drugs, such as the PDE4 inhibitors, have been linked to inhibition of leukocyte migration indirectly. PDE4 inhibitors, such as the small-molecule inhibitor Apremilast, act by supressing the PDE4-mediated degradation of cAMP. This leads to the attenuation of pro-inflammatory cytokine production and resolution of inflammation. The understanding of the mechanism of action behind this drug is very vague and it remains unknown why some patients respond poorly to it. Therefore, studying if and how current treatments for psoriasis affect leukocyte migration could allow us to develop better patient-specific treatments.

In this thesis, we hypothesise that skin targeting leukocytes interact with keratinocytes to orchestrate subsequent cell recruitment in a chemokine-dependent manner.

The aims of this thesis are to:

1. Develop an assay which would allow for the selection of leukocytes, more likely to migrate to a psoriatic lesion, from peripheral blood
2. Investigate the migratory potential of leukocytes and ability of psoriasis-associated chemokines to attract leukocytes in the context of psoriasis
3. Investigate the effects of different drugs for the treatment of psoriasis on leukocyte migration
4. Investigate chemokine-orchestrated leukocyte-stromal cell interactions in psoriasis

Chapter 2: Materials and methods

2.1 General reagents

Drug dissolving media: DMSO was purchased from Insight Biotechnology, UK;

EDTA: Ethylenediaminetetraacetic acid (EDTA) was purchased from Santa Cruz, USA;

FACS buffer: 50mL PBS without Ca^{2+} and Mg^{2+} was mixed with 250 µL FBS, 200 µL EDTA 0.5M;

HBSS: Hank's Balanced Salt Solution (HBSS) with calcium and magnesium, without phenol red was purchased from Lonza, UK;

Keratinocyte detachment media: HEPES BSS (N-2-hydroxyethylpiperazine-N-ethanesulfonic acid buffered Balanced Salt Solution), Trypsin/EDTA Solution (The Trypsin/EDTA ratio is 0.04%/0.03 %.) and TNS (Trypsin Neutralization Solution) was purchased from Promocell, Germany;

Keratinocyte freezing media: Freezing Medium Cryo-SFM was purchased from PromoCell, Germany;

Keratinocyte media: Growth Medium 2 was purchased from PromoCell, Germany and supplemented with $CaCl_2$ and PromoCell SupplementMix ;

Migration buffer: RPMI Roswell Park Memorial Institute Medium (RPMI) was mixed with 0.5% BSA;

Neutrophil isolation media: PBS without Ca^{2+} and Mg^{2+} was mixed with 1 mM EDTA;

Pan Buffer for T-cell isolation: 50mL PBS without Ca2+ and Mg2+ was mixed with 0.5% BSA and 100mM EDTA;

PBS: Dulbecco's Phosphate-Buffered Saline (DPBS) without magnesium and calcium was purchased from Gibco (ThermoFisher Scientific, UK);

Plastics: All tissue culture plastics used for experiments were purchased from VWR (USA) or Corning (UK). 1.5 ml microcentrifuge tubes were purchased from Eppendorf (UK). All tips were filter tips and purchased from starlabs (UK) or Rainin (UK). qPCR plates were purchased from Sigma Aldrich (USA). 9mL Vacuette tubes containing K_3 Ethylenediaminetetraacetic acid EDTA were purchased from Greiner-bio-one (UK);

RNase-free water: RNase free water was purchased from Invitrogen;

RPMI: Roswell Park Memorial Institute Medium (RPMI) 1640 was purchased from Thermo Fisher Scientific, USA;

T-cell media migration and culture: X-VIVO™ 15 Serum-free Hematopoietic Cell Medium was purchased from Lonza, UK;

Tissue digestion media: 0.9ml of HBSS was mixed with 1mg/ml of Collagenase D (Roche/Sigma Aldrich, USA) (skin and spleen only), 0.5mg/ml of Dispase II (Roche/Sigma Aldrich, USA) (skin and spleen only), 0.1mg/ml DNase I (Roche/Sigma Aldrich, USA).

2.2 Ethical approval

Participant samples were collected under three separate studies, all approved by relevant ethical committees (see below). Example of the Patient Information Sheet and consent forms are attached in the Appendix.

2.2.1 Study 1

Study name: Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto)inflammatory diseases

Sponsor Ref: MM04/6710

Ethics Ref: 04/Q1206/107

Ethical committee: Leeds (East) Research Ethics Committee

Nature of samples: Peripheral blood samples

Inclusion criteria: Subject not diagnosed with autoimmune or (auto) inflammatory diseases; subject is capable of understanding and signing an informed consent form

Exclusion criteria: Subject lacks capacity to give informed consent

2.2.2 Study 2

Study name: Psoriatic Disease Research (PDAR)

Sponsor: Leeds teaching hospitals trust (Sponsor Ref: DM15/382)

Ethics Ref: 16/HY/0086

Ethical committee: Yorkshire and the Humber-Leeds West Research Ethics Committee

Nature of samples: Peripheral blood samples

Inclusion criteria: Subject ≥ 16 years of age; subject is capable of understanding and signing an informed consent form; has a clinical diagnosis of psoriasis.

Exclusion criteria: Age less than 16 years; lack of capacity to give informed consent; pregnant

2.2.3 Study 3

Study name: Rheumatoid Arthritis Disease Research (RADAR)

Sponsor: University of Leeds (Sponsor Ref: RR09/9134)

Ethics Ref: RR09/9134
Ethical committee: The Leeds (West) Research Ethics Committee

Nature of samples: Peripheral blood samples

Inclusion criteria: Adults treated in the outpatient rheumatology service; subjects must be willing and able to sign appropriate Consent Form; has, or has had, at least one of the following e.g. inflammatory joint symptoms, clinical sings of inflammatory arthritis such as synovitis, imaging evidence of inflammatory arthritis including but not limited to synovitis, erosion, tenosynovitis

Exclusion criteria: Younger than 18 years of age; lack of capacity to give informed consent

2.2.4 Murine experiments

All animal experiments were undertaken following regulated ethical approval by local University of Leeds AWERB and Home Office, under project licence PA7CF4E75. Animal experiments were undertaken by Clive McKimmie who has a personal licence. Sample generated were then processed and analysed by myself.

2.3 Study subjects and controls

All studies were approved by the Leeds teaching Hospitals NHS Trust Medical Ethics Committee and informed consent was obtained from all subjects and healthy volunteers. For ethical approval details, please see section above and Appendix.

2.3.1 Patients with psoriasis

Patients with psoriasis used in this study were either untreated, or treated with drugs, such as Apremilast, Methotrexate, Secukinumab, Ustekinumab, Infliximab, Ixekizumab, at various stages of their treatment and with varying degree of treatment response (as indicated in results). The mode of action of these drugs can be found in table 1.1. Diagnosis of psoriasis was made via the examination of the skin lesion and its morphological properties by a dermatologist according to applicable guidelines[24]. The morphological features which reflect active skin lesions, include erythematous, silvery white scaly, sharply demarcated plaques. The inclusion/exclusion criteria and ethical approval for the recruitment of patients with psoriasis can be found in section 2.2.2.

2.3.1.1 Untreated patients with psoriasis

Patients with Psoriasis were recruited from the Leeds Centre for Dermatology at Chapel Allerton Hospital, Leeds. All patients had clinical features consistent with the diagnosis of psoriasis (well-demarcated, symmetric, and erythematous plaques with overlying silvery scale). Peripheral blood samples were obtained at presentation from patients with mild to severe psoriasis. The severity of psoriasis was measured via the Psoriasis Area and Severity Index (PASI), as described in chapter 1.1.3.

PASI score	Psoriasis severity
<10	Mild
10-15	Moderate
>15	Severe

Table 2.1. PASI score and Psoriasis severity

2.3.1.2 Treated patients with psoriasis

These are patients with psoriasis who were treated with Apremilast, Methotrexate, Secukinumab, Ustekinumab, Infliximab or Ixekizumab, prior to sample collection, and achieved a reduction of PASI score or complete clinical remission. The dosing schedule is as per the drug's UK licence/in

accordance with national guidelines, and all patients were stablished on a stable dose of treatment before sampling.

2.3.2 Disease control patients

Peripheral blood samples were obtained from patients with neutrophilic dermatoses as a pertinent group of exemplar diseases, that are neutrophil driven, poorly understood, and would make a relevant comparator to psoriasis (NDs). These were used as controls and include Behçet's disease, Pyoderma gangrenosum, and Hidradenitis Suppurativa. All patients had clinical features consistent with the diagnosis of NDs, including e.g. abnormally high neutrophil numbers, painful inflamed lesions, discoloured skin, fever, fatigue[90-92]. Further to this, peripheral blood samples were also obtained from patients with psoriatic arthritis. All patients had clinical features consistent with the diagnosis of psoriatic arthritis, as diagnosed by a rheumatologist. Diagnosis of psoriatic arthritis was made via joint and nail examination (for signs of swelling, tenderness, flaking), imaging tests (X-rays to help identify joint changes). The inclusion/exclusion criteria and ethical approval for the recruitment of patients with NDs and psoriatic arthritis can be found in section 2.2.2 and 2.2.3.

2.3.3 Healthy controls

Fresh blood samples used for chemotactic assays, flow cytometry, mRNA extraction, qPCR and co-culture experiments were obtained from healthy control subjects, working in the Wellcome Trust Brenner Building, St James University Hospital, Leeds. These subjects are referred to as 'non-psoriasis donors' or 'healthy donors' in this thesis. The inclusion/exclusion criteria and ethical approval for the recruitment of healthy controls can be found in section 2.2.1.

2.4 Sample processing

Peripheral blood (PB) samples (9-27mL in total) from patients with psoriasis (n=101), patients with NDs (n=9), patients with psoriatic arthritis (n=14) and non-psoriasis donors (n=12), were collected using 9mL Vacuette tubes (Greiner-bio-one) containing K_3 Ethylenediaminetetraacetic acid (EDTA). Patient blood was obtained at Chapel Allerton Hospital and in order to minimise inadvertent cell activation, was carefully transported to Welcome Trust Brenner Building, St. James University Hospital. Healthy blood was obtained at Clinical Sciences Building, St James University Hospital. All blood samples were processed within 1-3 hours of venepuncture.

2.4.1 Neutrophil isolation

Blood was obtained from either healthy donors or patients. 5mL of blood was transferred to a 14mL round-bottom polystyrene tube. A direct neutrophil isolation from human blood kit was used, as per the manufacturer's instructions (Stemcell Technologies, Canada). RapidSpheres and Isolation cocktail were added (50µL of each per mL of sample). The sample was incubated for 5 minutes at room temperature. Prior to the first round of magnetic separation (5 minutes), the volume of the sample was made up to 12mL with the recommended medium (Ca^{2+} & Mg^{2+} free Phosphate-buffered saline (PBS) (Thermo Fisher Scientific, USA) containing 1mM EDTA (Santa Cruz, USA). Following the first round of magnetic separation, a further 250µL of RapidSpheres were added to the sample. The sample was incubated for 5 minutes at room temperature, prior the final two rounds of magnetic separation (5 minutes per each round). Following isolation, neutrophils were resuspended in neutrophil media (Roswell Park Memorial Institute Medium (RPMI) 1640 (Thermo Fisher Scientific, USA) with 0.5% Bovine serum albumin (BSA) (Miltenyi, Germany)).

2.4.2 Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood

Blood was obtained from either healthy donors or patients with psoriasis. Peripheral blood mononuclear cells (PBMCs) were separated from blood using density gradient centrifugation over a sucrose density gradient layer. The blood was diluted 1:1 with room temperature Ca^{2+} & Mg^{2+} free PBS and 25mL of the mixture was layered onto 12.5mL of Ficoll-Paque Plus (Sigma-Aldrich, UK). This was centrifuged at 2400rpm for 20 minutes at room temperature (acceleration: 5; no brake). The PBMC layer was harvested and washed in 50mL of PBS (1800rpm, 15 minutes, with brake and acceleration on full). The PBMCs were re-suspended in 10mL PBS and counted by using dead cell exclusion dye - trypan blue (Thermo Fisher Scientific, USA), a haemotocytometer and a light microscope. PBMCs were washed again at 1500rpm for 10 minutes.

2.4.3 T-cell isolation

Total CD3+ T-cell lymphocytes were isolated from PBMCs or directly from whole blood. Total CD4+ T-cell lymphocytes were isolated from PBMCs.

2.4.3.1 CD3+ T-cell isolation from PBMCs

PBMCs were resuspended in 40µL of Pan Buffer (PBS pH 7.2 (50mL), 0.5% BSA (2.5mL), 100mM EDTA (1mL)) per 10^7 cells. The cells were incubated with 10µL of Pan T Cell Biotin-Antibody Cocktail (Miltenyi, Germany) per 10^7 total cells for 5 minutes at 4°C. Pan buffer was added to the cells (30µL of buffer per 10^7 total cells). The cells were incubated with 20µL of Pan T-Cell MicroBead Cocktail (Miltenyi, Germany) per 10^7 total cells for 10 minutes at 4°C. The volume was made up to 500µL prior LS column (Miltenyi, Germany) magnetic separation. CD3+ fraction was collected and resuspended in RPMI media with L-glutamine and 0.5% BSA, as per manufacturer's instructions.

2.4.3.2 CD3+ T-cell isolation from whole blood

Blood was obtained from either healthy donors or patients. 5mL of blood was transferred to a 14mL round-bottom polystyrene tube. A direct CD3+ T-cell isolation from human blood kit was used, as per the manufacturer's instructions (Stemcell Technologies, Canada). RapidSpheres and Isolation cocktail were added (50µL of each per mL of sample). The sample was incubated for 5 minutes at room temperature. Prior to the first round of magnetic separation (5 minutes), the volume of the sample was made up to 12mL with the recommended medium (Ca^{2+} & Mg^{2+}-free PBS). Following the first round of magnetic separation, a further 250µL of RapidSpheres were added to the sample. The sample was incubated for 5 minutes at room temperature, prior the final two rounds of magnetic separation (5 minutes per each round). CD3+ fraction was resuspended in RPMI media with L-glutamine and 0.5% BSA.

2.4.3.3 CD4+ T-cell isolation from PBMCs

PBMCs were resuspended in 1mL of Recommended Medium (PBS pH 7.2, 2% Fetal Bovine serum (FBS), 1mM EDTA) per $5x10^7$ cells. The cells were added to a 5mL polystyrene round-bottom tube and incubated with 50µL/mL of Isolation Cocktail (Stemcell Technologies, Canada) per $5x10^7$ total cells for 5 minutes at room temperature. RapidSpheres (50µL/mL) were added to the mixture and the volume was made up to 2.5mL with Recommended Medium prior to magnetic separation. CD4+ fraction was collected and resuspended in RPMI media with L-glutamine and 0.5% BSA.

2.4.4 Activation of T-cells

T Cell Activation/Expansion Kit (Miltenyi, Germany) was used for the activation of T cells. This kit works by combining Anti-Biotin MACSiBead Particles and biotinylated antibodies to mimic antigen-presenting cells and activate resting T cells. Prior the activation, $1x10^8$ Anti-Biotin MACSiBead Particles were loaded with CD2-Biotin and CD28-Biotin in a total volume of 1mL buffer (PBS pH 7.2, supplemented with 0.5% BSA and 2 mM EDTA) and stored at 4°C, as per manufacturer's instructions. The loaded Anti-Biotin MACSiBead Particles were resuspended and 25µL (2.5×10^6 loaded Anti-Biotin MACSiBead Particles) per 5×10^6 T-cells were washed in 200µL culture medium (RPMI 1640 supplemented with 10% Foetal calf serum (FCS)) (300g for 5 minutes). The beads were resuspended in 100µL fresh culture medium and added to the freshly isolated T-cells resuspended at a density of 5×10^6 cells per 900 µL of culture medium. The mixture was added to a suitable cell culture vessel at a density of 5×10^6 cells per mL per cm^2 and incubated at 37°C, 5% CO_2 for up to 72h. Incubation times are indicated on the appropriate figures.

2.4.5 Stimulation of Neutrophils

Where indicated in figures, freshly isolated neutrophils were activated via Toll-like receptor (TLR) 4-stimulation with Lipopolysaccharide (LPS) (Thermo Fisher Scientific, USA). LPS at a concentration of 15ng/mL was added to the cells and incubated at 37°C for 30 minutes in a CO_2 incubator. Neutrophils were resuspended in fresh media prior to downstream use.

2.5 Mice

All mice were wild-type female C57BL/6J mice and bred in-house at the SBS at the University of Leeds. Mice were housed at SBS under pathogen-free conditions and had access to food and water. All animal work was conducted with Dr Clive McKimmie in accordance with the United Kingdom Home Office regulations and carried out under the authority of appropriate project and personal license.

2.5.1 Imiquimod mouse model

The Imiquimod mouse model was utilised as it was first described by van der Fits et al. 2009. In the morning of Day 0, mice aged 8 weeks had a small section of their back skin gently denuded

by trimming. In the afternoon of Day 0, mice were anesthetised via inhalation and treated with Imiquimod. Mice were treated daily with 62.5mg 5% imiquimod cream (Aldara™, Meda, UK), on the denuded back skin area and back feet, for a total of 4 days. Following fur removal, control mice were left untreated.

2.5.2 Preparation of Apremilast and mouse dosing

As per previous publications (McCann et al. 2010), mice were dosed with 25 mg/kg of Apremilast via oral gavage. 25mg of Apremilast was purchased from Generon (Slough, UK) and was reconstituted in 500µL of dimethyl sulfoxide (DMSO) (Insight Biotechnology, Wembley) to achieve a stock concentration of 0.05mg/µL. The Apremilast stock was stored at -20°C in aliquots. A fresh aliquot was dissolved into carrier solution prior to dosing, daily. The carrier was prepared fresh on a day-to-day basis and consisted of 0.5% carboxymethyl cellulose (CMC) (Sigma, UK), 0.25% Tween 80 (Scientific Laboratory Supplies, UK), 50 mL clean water. On the night prior to dosing, 50mL of clean water was poured into a 50mL Corning (Liversedge, UK) centrifuge tube and 0.25g of CMC was evenly distributed onto the water surface. This was carefully placed in a 4°C fridge overnight, which allowed for the CMC to dissolve sufficiently. An alternative CMC dissolving strategy was also tested, whereby 0.25g of CMC was divided into three parts and each part was gradually added to three separate parts of 2mL clean water (whilst stirring) until dissolved. The three parts (6mL in total) were then added to 44mL of clean water and placed in a fridge overnight. 0.125mL of Tween 80 was gradually added, whilst mixing with a metal stirrer, to 2mL of the now completely dissolved CMC/water mix. When necessary, more of the CMC/water mix was added until Tween 80 was completely dissolved. This was then mixed back in with the remainder of the CMC/water mix. On the day of dosing, for a mouse of 20g, 10µL of Apremilast stock (0.5mg Apremilast) was slowly added to 200µL of carrier solution, whilst mixing. Effort was applied, for minimal precipitation to occur. The Apremilast + carrier mix was orally given to mice daily, for four days, prior to treatment with Aldara. A group of control mice received carrier alone (no drug) + Aldara™, whereas a second control group received no treatment at all (no Aldara™, no Apremilast, no carrier). Every treatment group in the *in vivo* experiment included 5 mice.

2.5.3 Disease evaluation and mice monitoring

Mice were weighted daily and weight loss greater than 20% was considered as cut-off for cull, as per Home office regulations. Mouse skin was assessed daily for psoriasiform inflammation

and quantified using a modified PASI score, developed by Dr Kave Shams. This scoring system took into account the degree of skin thickening, erythema (redness) and skin scaling, but did not include percentage of body part covered (as inflammation induced at a discrete site).

2.5.4 Tissue processing

Mice were culled via a rising concentration of carbon dioxide. Dissected tissues included skin from foot, skin from back and femur. Skin from foot was placed in 1mL of RPMI 1640 + 10% FCS medium in 1.5 mL Eppendorf tubes (Eppendorf, Germany), prior to digestion (detailed in section 2.5.7). Back skin tissues were halved. For the purpose of microscopy, one half was placed in 1mL of 10% Formalin (Sigma, UK) in a 1.5mL Eppendorf tube for 24-72 hours, then in 70% ethanol (Sigma Aldrich, USA). These were then sourced out to technical staff at the University of Leeds, Welcome Trust Brenner Building (Level 4) for processing, embedment, sectioning and H&E staining. The other half was placed in 0.5mL RNAlater (Sigma, UK) in a 1.5mL Eppendorf tube at 4°C for 72 hours (to prevent RNA degradation) and then at -80°C for long-term storage.

2.5.5 Skin digestion

In order to quantify neutrophil influx into mouse skin, the skin had to be digested and cells extracted. Digestion of skin was done in digestion medium, which consisted of the following reagents, per sample:

- 900µL Hank's Balanced Salt Solution (HBSS) (Lonza, UK)
- 100µL collagenase D (10 mg/mL stock, 1 mg/mL final concentration) (Sigma Aldrich, USA)
- 50µL Dispase II (10 mg/mL stock, 0.5 mg/mL final concentration) (Sigma Aldrich, USA)
- 8µL DNase I (12.5 mg/mL stock, 0.1 mg/mL final concentration) (Sigma Aldrich, USA)

The obtained foot skin samples were transferred from RPMI+ 10% FCS medium to digestion medium in 1.5 mL Eppendorf tubes. Once in the digestion medium, the skin was chopped into small pieces, using springbow dissecting scissors. All samples were handled on ice and at a fast pace. When all chopped, samples were incubated shaking 1400RPM at 37°C for 50 minutes in a ThermoMixer F1.5 (Eppendorf, Germany). Samples were transferred on ice and 500µL of

RPMI+ 10% FCS was added to them. Each digested sample was run through a 70µM cell strainer (Scientific Laboratory Supplies, UK). Eppendorf tubes were washed once with media to get all bits of skin out and media was added to the corresponding cell strainer. Skin remnants were mashed with a 5mL syringe plunger (B. Braun Omnifix, Germany) into a 50mL centrifuge tube and the strainer was flushed using 1 mL of RPMI+ 10% FCS medium. Cells were spun down for 5 minutes at 300g, 4°C and prepared for staining with antibodies (staining procedure is outlined in section 2.8.1.1).

2.5.6 Extraction of bone marrow cells

Bone marrow cells were used as a control in the flow cytometric analysis of mouse skin and for achieving compensation. Bone marrow was extracted from the femur of mice. In short, an incision was made on either side of the spinal cord and femur was cut out. Skin, muscle tissue and fat were removed from the femur of mice until a clear view of the bone was achieved. The femur bone was extracted by bending back the knee joint. The bone was cut with sharp, ethanol soaked scissors on both ends to expose the marrow. The bone marrow cells were flushed with RPMI+ 10% FCS medium using a 5mL syringe and a 26G needle (BD, Ireland). Cells were dissociated by gently pipetting up and down with a 5mL pipette. Cells were spun down for 5 minutes at 300g, 4°C and resuspended in 1mL of red cell lysis buffer for 2 minutes at room temperature. Cells were then topped up to 5mL with FACS buffer (50ml PBS, 250 µL FBS, 200 µL EDTA 0.5M), passed through a 70µM cell strainer, counted, spun down, and resuspended in FACS buffer at 1M/mL.

2.5.7 Acquisition and analysis of H&E stained sections

Images of the H&E stained back skin tissue slides were captured with an Infinity 1 camera (Lumenera, Canada). Images of each sample were taken at three random areas of the slide with 20x and 40x magnification. These were then imaged on and analysed using Fiji ImageJ (NIMH, USA). The size of resting, uninflamed epidermis was used - 10 µM – to set a default pixel per inch ratio (measurement scale). Therefore, images captured at 10x magnification were analysed with a measurement scale of 2.9 pixels per inch; images captured at 20x magnification were analysed with a measurement scale of 5.8 pixels per inch. Analysis of epidermal and dermal thickness was carried out on images captured at 10x magnification, whereas counting of neutrophils was done on images captured at 20x magnification. Counting of neutrophils could only be done on images captured at 20x magnification, as nucleus was not

visible at a lower magnification. A cell was regarded as a neutrophil if it looked like the cell shown in figure 4.14. Prior to counting, an area with the size of 200x150inch was selected in the centre of each image and neutrophils were counted within the selected area. Neutrophils were counted and epidermal/dermal thickness was measured in three images (each captured at a random area of a given slide) per sample. An average number of the three values was generated to represent a given sample. Analysis was done on Microsoft Excel (Microsoft, Seattle, USA).

2.6 Cell culture

Cell culture was performed in sterile conditions, using aseptic technique in a category two tissue culture hood (NuAire, UK). Primary cells were cultivated in a humidified incubator (Panasonic, The Netherlands) at 37°C in 5% CO_2 atmosphere. Experiments were repeated using cells from at least 2 separate cell donors.

2.6.1 Primary cell culture: Keratinocytes (KC)

Primary human keratinocytes isolated from the epidermis of healthy adult skin donors were purchased from PromoCell, Germany. These cells shall be referred to as Normal Human Epidermal Keratinocytes (NHEK). NHEK cells arrived on dry ice in cryopreserved vials (each vial contained >500,000 NHEK cells) and were stored in liquid nitrogen or seeded immediately. These cells were used in passage 3 (P3), after thawing and cultivation (see below).

2.6.1.1 Primary cell culture: thawing of keratinocytes

NHEK cells were seeded and cultured in accordance to manufacturer's instructions (PromoCell, Germany). Following removal from dry ice and a 2-minute thawing procedure (37°C water bath) and sterilisation (70% ethanol), NHEK cells were transferred to a sterile cell culture flask (T-25 size), containing pre-warmed medium. The NHEK cell culture medium – Growth Medium 2 (Ready-to-use) - was purchased from PromoCell, Germany and its contents (after supplementation with $CaCl_2$ and PromoCell SupplementMix) can be found in table 2.2. The NHEK cells were placed in an incubator and were allowed to adhere to the cell culture flask surface (approximately 16 hours). After becoming adherent, the medium was changed.

Table 2.2. Final supplement concentrations (after addition to the medium)

Bovine Pituitary Extract	0.004 mL/mL
Epidermal Growth Factor (recombinant human)	0.125 ng/mL
Insulin (recombinant human)	5 µg/mL
Hydrocortisone	0.33 µg/mL
Epinephrine	0.39 µg/mL
Transferrin (recombinant human)	10 µg/mL
$CaCl_2$	0.06 mM

2.6.1.2 Primary cell culture: subcultivation of keratinocytes

Upon reaching confluency of over 90%, NHEK cells were subcultivated. The PromoCell DeatchKit (Cat. Number: C-41200) was used for the subcultivation of NHEK cells. The PromoCell DetatchKit consists of three components: HEPES BSS (N-2-hydroxyethylpiperazine-N-ethanesulfonic acid buffered Balanced Salt Solution), Trypsin/EDTA Solution (The Trypsin/EDTA ratio is 0.04%/0.03%.) and TNS (Trypsin Neutralization Solution). HEPES BSS contains 30 mM HEPES, D-Glucose, NaCl, KCl, Na-Phosphate and Phenol Red. TNS contains 0.05% Trypsin Inhibitor from soybean and 0.1% BSA. The DetatchKit was brought to room temperature. The NHEK medium was aspirated and NHEK cells were washed in 100µl of Hepes BSS solution per cm^2 of vessel surface and the vessel was agitated carefully for 15 seconds. The Hepes BSS was aspirated and 100µl of Trypsin/EDTA solution per cm^2 of vessel surface was added. NHEK cells were left at room temperature until detached (no longer than 10 minutes). TrypsinNeutralisation Solution was added (100µl per cm^2 of vessel surface) and the NHEK cell suspension was transferred to a centrifugation tube. NHEK cells were spun down for 3 minutes at 220xg. The supernatant was discarded and 1mL of NHEK growth medium was added. The resuspended cells were plated in a T-75 cell culture flask and placed in a humidified incubator at 37°C in 5% CO_2 atmosphere.

2.6.1.3 Primary cell culture: freezing of keratinocytes

Upon reaching confluency of 90%, NHEK cells were detached, resuspended in Freezing Medium Cryo-SFM (PromoCell, Germany) at a concentration of 1.5 million cells/mL and aliquoted into cryovials. Cryovials were frozen at a rate of 1°C per minute in a -80°C freezer and were later transferred to liquid nitrogen for long term storage. Freezing Medium Cryo-SFM is a protein-

free, defined and animal component-free cryopreservation medium. The optimized formulation is based on methylcellulose, DMSO and other cryoprotectants (proprietary mixture, composition undisclosed by PromoCell) for the cryopreservation of primary cells.

2.6.2 Primary cell culture: leukocytes

Primary human leukocytes (neutrophils or/and T-cells) were isolated from the peripheral blood of patients with psoriasis, disease control group patients or healthy donors (as described in section 2.3). Following isolation these cells were either lysed (using the lysis buffer contained in the RNA micro kit (Life Technologies, UK) with 1% β-mercaptoethanol (Sigma Aldrich, USA) and stored at -80°C for future mRNA extraction; used in a transwell chemotactic assay or prepared for co-culture with keratinocytes. If to be co-cultured with KCs, leukocytes were resuspended in Growth Medium 2 (Ready-to-use) at a concentration of 1mL per 1 million cells. 5000 Leukocytes (Neutrophils or T-cells) were added to a single well of keratinocytes at 80% confluency in a 48-well plate.

2.6.2.1 Neutrophil treatment with Apremilast

Apremilast is a Phosphodiesterase-4 inhibitor, which is used for the treatment of mild-to-severe psoriasis. Apremilast was purchased from Generon (UK) and was used for the *ex-vivo* pre-treatment of neutrophils (indicated in appropriate figures). Apremilast was dissolved in DMSO and serially diluted in neutrophil medium (RPMI 1640 with 0.5% BSA) to yield a wide range of concentrations: 0.1μM, 1μM, 10μM, 100μM. The freshly isolated neutrophils were centrifuged (300g for 4 minutes) and resuspended in the appropriate Apremilast-containing medium at a density of 0.5 million cells per mL. 500μL of neutrophils were placed in a 24-well plate at a density of $2.5x10^5$ cells/well and were incubated in a CO_2 incubator for a duration of 120 minutes.

2.6.3 Primary cell culture: KC-leukocyte co-cultures

Co-cultures were placed in a humidified incubator and were maintained for not longer than 24 hours. Co-culture medium was collected and stored at -80°C; wells were washed with Growth Medium 2 (Ready-to-use) and fresh media was given to the KCs. The plate, with now leukocytes removed, was placed in a humidified incubator for a further 24-hour period. The supernatant

was collected and stored at -80°C; keratinocytes were lysed using the lysis buffer contained in the RNA micro kit (Life Technologies, UK) with 1% β-mercaptoethanol (Sigma Aldrich, USA) and stored at -80°C for later mRNA extraction.

2.7 Cell migration assay

Neutrophil and T-cell migration was measured using a transwell chamber assay. The transwell chambers (Corning, United States) utilised had the following specifications:

- 6.5 mm diameter of transwell insert (24-well plate format).
- Polycarbonate filters.
- 3µm or 5µm pore size for neutrophil or T-cell migration assays, respectively.

2.7.1. Neutrophil transmigration

Neutrophils were isolated from fresh peripheral blood (as per section 2.4.1) from either healthy donors or patients. Neutrophils were either treated with Apremilast (as per section 2.6.2.1) prior to the assay or readied for transmigration directly after isolation.

2.7.1.1 Chemokine preparation

Chemokines (CXCL8, CCL2 and CXCL16 for neutrophil chemotactic assays) were purchased from Peprotech (UK) and resuspended in neutrophil medium at a stock concentration of 20µg/mL. Dilutions of the chemokine stock were made in neutrophil medium and the dilutions used were 0 ng/mL, 10 ng/mL, 50 ng/mL, and 200 ng/mL.

2.7.1.2 Supernatant preparation and Anti-CXCL8 treatment

Supernatants derived from T-cell-conditioned keratinocytes were utilised as chemoattractive media in several experiments (indicated in appropriate figures). The supernatants were diluted with neutrophil medium at a ratio of 1:1. Where indicated, supernatants were treated with an anti-CXCL8 antibody (R&D systems, UK). The CXCL8 antibody was reconstituted in sterile PBS at a stock concentration of 0.5 mg/mL (as per manufacturer's instructions); aliquoted and stored long term at -80°C. It was determined that a concentration of 1µg/mL anti-CXCL8 antibody was sufficient to inhibit CXCL8-driven chemoattraction of neutrophils. Therefore, prior to the

transwell assay, anti-CXCL8 at 1µg/mL was added to the appropriate supernatants and placed in a CO_2 incubator for 30-60 minutes.

2.7.1.3 Neutrophil transwell assay

Following isolation from blood or pre-treatment with Apremilast, neutrophils were centrifuged (300g for 4 minutes) and resuspended in non-Apremilast-containing neutrophil medium at a concentration of 5 Million cells per mL. The chemokine-containing medium or supernatants were prepared sufficient for every reaction to be performed in a triplicate or duplicate (technical replicates). The transwell inserts were removed from the wells by using a sterile pair of forceps and 600µL of chemokine solution/supernatant, per well, was added in the bottom of the transwell plate, as per manufacturer's instructions. The plate was incubated at 37°C for 5 minutes and the transwell inserts were placed back onto the wells. This was followed by a further 5-minute incubation at 37°C. 100µL of cell suspension, as per manufacturer's instructions, (0.5 Million cells) were pipetted onto the top of the transwell inserts and were incubated at 37°C for 60 minutes.

2.7.2 T-cell transmigration

Following isolation from blood, T-cells were centrifuged (400g for 5 minutes) and resuspended in X-VIVO™ 15 Serum-free Hematopoietic Cell Medium (Lonza, UK). The remainder of this protocol is identical to the section above (2.7.1.3) with the following exceptions:

1. Chemokines used, included: CCL5; CCL18, CXCL10, CCL20, CCL17, CCL27, CXCL16, CX3CL1, CCL19. The chemokine stock was made up in the X-VIVO 15 media with 0.5% BSA added to it. X-VIVO 15 without BSA was used for the chemokine dilutions.
2. In several experiments (where indicated), instead of chemokine solution, supernatants derived from Neutrophil-conditioned keratinocytes were used as chemoattractive media. The supernatant was diluted 1:1 with X-VIVO 15.
3. The incubation time during transmigration was 90-150 minutes.

2.7.3 Counting of migrated cells and analysis

Following incubation, the contents of the top well (cells that have not migrated) were discarded of and filters were removed. The contents of the bottom well were collected and transferred to a 2mL Eppendorf; the well was then washed with PBS and the contents added to the respective Eppendorf tube. The Eppendorf tubes were centrifuged at 300g for 4 minutes; supernatant removed; cells resuspended in ice-cold PBS and counted using dead cell exclusion dye trypan blue. Cells were counted either manually using a light microscope (ZEISS, Germany) and a haemocytometer or using a Biorad automated cell counter (California, USA). Cells were then either discarded or resuspended in media for downstream use (co-culture with keratinocytes). To analyse migration, the number of migrated cells was divided by the number of total cells placed onto the top chamber. This was then multiplied by 100 to obtain percentage of migrated cells.

2.8 Flow Cytometry

Flow cytometric analysis was performed using Cytoflex S (Beckman Coulter, USA) or Cytoflex LX (Beckman Coulter, USA). Cytoflex S was used for all of the experiments, but the calcium flux assay, which was performed on Cytoflex LX. This was due to the limitations of the Cytoflex S machine – no 1.5mL Eppendorf tubes could be utilised. Flow cytometry data analysis was performed using FlowJo (BD, Switzerland) software, post capture.

2.8.1 Analysis of the purity of cell isolates

To confirm that cell isolation was successful, a flow cytometry-based assay was utilised. This was performed for every cell isolation kit used, using a universal protocol. The difference in every assay was the antibody staining mix, as the purity of different cells types was being assayed. The antibody staining mix for every cell type can be found in section 2.8.1.2.

The following cells and controls were used for each isolation kit purity assay:

- PBMCs, CD3+ and CD3 - fractions (Human CD3+ T cell isolation from PBMCs) - CD3+ cell isolate purities of 94% were confirmed;
- CD15+CD16+ cells and PBMCs (Human Neutrophil isolation from whole blood) - CD15+CD16+ cell isolate purities of 96.7% were confirmed;

- CD3+ fraction and PBMCs (Human CD3+ T-cell isolation from whole blood) - CD3+ cell isolate purities of 94% were confirmed;
- CD4+ fraction and PBMCs (Human CD4+ T-cell isolation from PBMCs) – CD4+ cell isolate purities of 94% were confirmed.

2.8.1.1 Cell staining protocol

Cells were transferred to fluorescence-activated single cell sorting (FACS) tubes (1×10^6 cells in each tube) and washed in 300µL of FACS buffer (400g for 5 minutes for T cells; 300g for 4 minutes for neutrophils). Cells were resuspended in 50µL FC block solution (BioLegend, United States) (5µL FcR blocking reagent + FACS buffer per tube, as per manufacturer's instructions) and incubated for >5minutes on ice. Cells were incubated with 50µl of the antibody mastermix (5µl of each antibody; made up to 50µl with FACS buffer) for 30 min at 4°C in the dark and washed twice in 300µl of PBS (400g for 5 minutes for T cells; 300g for 4 minutes for neutrophils, 4°C). Cells were resuspended in 100 µL dead cell stain mix (Thermo Fisher Scientific, United States) (2 µL eFlour 780 + 1000 µL PBS) per tube and incubated for 10 minutes at room temperature. Cells were washed twice in 300µL of FACS buffer (400g for 5 minutes for T cells; 300g for 4 minutes for neutrophils). Cells were resuspended in 350µl of FACS buffer before FACS analysis. Unstained PBMCs were used as a negative control.

2.8.1.2 Antibody master mix

Analysis of the purities of neutrophils, isolated with EasySep Direct Human Neutrophils isolation kit, by FACS was performed using the following antibody mastermix:

Fluorophore	Antibody	Clone	Manufacturer
PE-Cy7	Anti-human CD19	HIB19	BioLegend, UK
FITC	Anti-human CD3	UCHT1	BioLegend, UK
V500	Anti-human CD16	3G8	BioLegend, UK
APC	Anti-human CD15	MMA	Thermo Fisher Scientific, UK

Analysis of the purities of T-cells, isolated with Pan T cell isolation kit, Miltenyi/ EasySep Direct Human T-cell isolation kit, StemCell, by FACS was performed using the following antibody mastermix:

Fluorophore	Antibody	Clone	Manufacturer
APC	Anti-human CD19	HIB19	BioLegend, UK
FITC	Anti-human CD3	UCHIT1	BioLegend, UK
V450	Anti-human CD56	5.1H11	BioLegend, UK

Analysis of the purities of T-cells, isolated with EasySep Human CD4+ T-cell isolation kit, StemCell, by FACS was performed using the following antibody mastermix:

Fluorophore	Antibody	Clone	Manufacturer
PerCP-Cy5.5	Anti-human CD19	HIB19	BioLegend, UK
Alexa Fluor-700	Anti-human CD4	RPA-T4	BioLegend, UK
V450	Anti-human CD56	5.1H11	BioLegend, UK

2.8.2 Analysis of neutrophil intracellular calcium transients

2.8.2.1 Fluo-4

To analyse the fluctuation of intracellular calcium concentration in neutrophils, the green-fluorescent calcium indicator, Fluo-4, was utilised. Fluo-4 is an acetoxymethyl (AM) ester – it will only fluorescence (on FITC channel; 494/506 nm) once it has been taken into cells and the dye is cleaved by esterase. Fluo-4 is essentially non-fluorescent without Ca^{2+} present, but the fluorescence increases at least 100 times upon Ca^{2+} binding. Fluo-4 was purchased from Thermo Fisher Scientific (UK). 1mM stock solution was prepared, by adding 45.6uL of anhydrous DMSO to 50ug vial of Fluo-4, aliquoted and froze down to -20°C for long-term storage.

2.8.2.2 Staining protocol

For each experiment, 1mM of Fluo-4 stock was diluted down to a final concentration of 2µM in HBSS (with Calcium and magnesium, without phenol red) (Scientific Laboratory Supplies, UK). Neutrophil were isolated from fresh blood (as described in section 2.4.1) and were pre-treated with 100uM Apremilast (as described in section 2.6.2.1) or left untreated (control). Following pre-treatment, neutrophils were washed twice in HBSS (300g for 4 minutes) and were resuspended at $3x10^6$/mL in HBSS containing Fluo-4 at 2µM. Following a 45-minute incubation period at 37°C, the neutrophils were washed twice in HBSS (300g for 4 minutes). Neutrophils were resuspended in HBSS and allowed to rest for 30 minutes in the dark at room temperature. Cells were washed and resuspended in HBSS at $1x10^6$ in 300µL per Eppendorf tube (1.5mL). Stimulator solutions were prepared in HBSS, including ionomycin (Cambridge Bioscience, UK) and CXCL8 (Peprotech, UK).

2.8.2.3 Cytoflex LX set-up

On the Cytoflex LX, time was set as a recorder parameter; storage set number of events was set to >1000000. Total time recorded was 10 to 13 minutes and addition of stimulants was done in the following order:

1. Baseline value for Fluo-4 was recorded for 30-60 seconds;
2. 50 ng/mL of CXCL8 were added in real-time and the calcium flux was recorded for 5-6 minutes or until it returned to baseline;
3. 2µM of Ionomycin was added to generate a maximum calcium flux, which would serve as a positive control.

2.8.2.4 Analysis of calcium flux data post-capture

The post-capture analysis was done using the kinetics platform of the FlowJo software. Multiple time intervals (1 second intervals) were created and the emission values for each time interval were recorded into GraphPad Prism (San Diego, USA) to generate a graph.

2.8.3 Analysis of CXCR2 expression by neutrophils

Neutrophil were isolated from fresh blood (as per section 2.4.1) and were treated with 100uM Apremilast (as per section 2.6.2.1) or left untreated (control). The staining protocol from section

2.8.1.1 was followed with the following antibodies: Anti-CXCR2-PE (Clone: REA208) (Miltenyi, Germany); Anti-CD15-APC (Clone: MMA) (Thermo Fisher Scientific, USA). When staining with anti-CXCR2, protocol from section 2.8.1.1 had to be amended to accommodate manufacturer's instructions and only 2µL per test were used.

2.8.4 CD4+ T cell subset characterisation

T-cells were isolated from fresh blood (as per section 2.4.3.2) and were allowed to migrate towards CCL20 in a chemotaxis assay (as per section 2.7.2) for 90 minutes. CCL20-responsive T-cells were collected and stained for the following markers (as per section 2.8.1.1): CD3, CD4, CCR6 and RORγt. Due to a clash in fluorophore antibody colours the stains had to be performed in duplicates. The cells were divided into two separate tubes, one tube was stained with antibodies against CD3 and CCR6, whereas the one against CD4 and RORγT. In order to stain for intracellular markers, such as RORγt, cells had to be permeabilised and fixed. This was achieved using a transcription factor staining solution kit (Invitrogen, UK). In short, after staining for cell surface antigens (e.g. CD4, CCR6), as per section 2.8.1.1, cells were resuspended and 1000 µL of Foxp3 Fixation/Permeabilization working solution was added to each tube. The tubes were incubated for 45 minutes at 4°C (protected from light) and were then washed twice in 2 mL of 1X Permeabilization Buffer. Prior to addition of RORγt and T-bet staining antibodies, the cells were resuspended in 100µL of 1X Permeabilization Buffer. Staining antibodies were added and cells were incubated at 4°C for 30 minutes in the dark and were then washed two times with 2 mL of 1X Permeabilization Buffer. Prior to flow cytometric analysis, cells were resuspended in FACS buffer.

Antibody fluorophores used for Th17 characterisation (tube 1):

Fluorophore	Antibody	Clone	Manufacturer
PE-Cy7	Anti-human CCR6	G034E3	BioLegend, UK
FITC	CD3	UCHT1	BioLegend, UK

Antibody fluorophores used for Th17 characterisation (tube 2):

Fluorophore	Antibody	Clone	Manufacturer
Alexa Fluor-700	Anti-human CD4	RPA-T4	BioLegend, UK
PerCP	Anti-human RORγt		BioLegend, UK

2.8.5 Analysis of Ly6G^{hi}Ly6C^{int} cell frequency in the skin of mice

Once extracted from the skin or bone marrow, cells were transferred into FACS tube. The staining protocol from section 2.8.1.1 was followed with the following adjustments:

1. The FC receptor block solution was substituted by a mouse equivalent: Purified Rat anti-Mouse CD16/CD32 (BD, Switzerland)
2. The cell viability dye used was substituted with DRAQ7(Cell signalling, The Netherlands). At the end of the surface marker staining step, cells were washed once (300xg, 5min, 4°C) and resuspended in DRAQ7 staining mix (1µL of DRAQ7 in 50µL FACS buffer, per sample). Cells were incubated for 5 minutes at room temperature, then placed on ice and protected from light for 20 minutes. Cells were then topped up to 300µL with FACS buffer, prior to Flow cytometric analysis.
3. Two antibody combinations were tested during optimisation stages (outlined below), but Combination 1 was used in the main experiment.

Combination 1

Fluorophore	Antibody	Clone	Manufacturer
PE-Cy5	Anti-mouse CD45	30-F11	BioLegend, UK
PE	Anti-mouse Ly6C	HK1.4	BioLegend, UK
PE-Cy7	Anti-mouse CD11b	M1/70	BioLegend, UK
BV421	Anti-mouse Ly6G	1A8	BioLegend, UK

Combination 2

Fluorophore	Antibody	Clone	Manufacturer
PB	Anti-mouse CD45	30-F11	BioLegend, UK
PE	Anti-mouse Ly6C	HK1.4	BioLegend, UK
PE-Cy7	Anti-mouse CD11b	M1/70	BioLegend, UK
APC	Anti-mouse Ly6G	RB6-8C5	TONBO Biosciences (USA)

2.9 RNA extraction

2.9.1 RNA extraction from cells

Total RNA was extracted from adherent (keratinocytes) or non-adherent (T-cells; neutrophils) cells, using the Purelink RNA micro kit (Thermo Fisher Scientific, USA), as per manufacturer's instructions. Prior to lysis, culture medium was removed from the adherent cell monolayer and cells were washed gently in PBS. Non-adherent cells were washed once in PBS and a cell pellet was obtained via centrifugation (300g for 5 minutes) prior to cell lysis. PBS was removed and 350µL of RNA Lysis Buffer with 1% β-mercaptoethanol were added directly onto the cell layer/pellet. The buffer was pipetted vigorously up and down, and samples were then centrifuged using QIAshredders (Qiagen, UK) to ensure complete homogenisation. Following homogenisation, 1 volume of 70% ethanol was added to each sample and mixed well via vortexing to disperse precipitation. The mix was then transferred to a PureLink™ Micro Kit Column (with the Collection Tube) and spun down for 60 seconds at 12,000rpm, to ensure RNA binding to the column. The column was washed once in Wash buffer I (12 000rpm for 60s), placed in a new collection tube and In-column DNase I Treatment was carried out (15 minutes at room temperature), to ensure trace DNA removal and no SYBR green binding. The DNase reaction mix was prepared in an RNase-free tube and consisted of 10µL DNase and 70µL of reaction buffer, per sample. Samples underwent one further wash with 350µL of Wash Buffer I and two washes with 500µL of Wash buffer II, each at 12,000rpm, 15 seconds. The column was then centrifuged at 12,000rpm for 60 seconds in a column drying step, after which it was placed in a recovery tube. The purified RNA was eluted in 15µL nuclease-free water. The concentration of eluted RNA was measured by using a Nanodrop (Spectrophotometer, ND-1000), which measures optical density.

2.9.2 RNA extraction from tissues

All tissue samples were defrosted on ice and transferred from RNAlater to a 2mL tube with two stainless steel beads (Qiagen, UK) and 1mL Trizol reagent (Qiagen, UK). Skin tissues required two beads as it is a fibrous type of tissue and therefore more difficult to lyse. The tubes were then shaken on a Tissue Lyser (Qiagen, UK) at 50Hz for 10 minutes until complete tissue lysis was achieved. The, now homogenised, tissue samples were transferred to 1.5mL Eppendorf tubes and mixed with 200µL chloroform (VWR International, Germany). These were then

inverted (but not vortexed, as this may lead to the release of soluble genomic DNA) 15 times to allow for thorough mixing of the solutions. This was followed by centrifugation of the samples at 12,000g for 15 minutes at 4°C. This allowed for the mixture to become separated into a lower red phenol-chloroform layer and a colourless upper aqueous phase. 500µL of the aqueous phase, containing the RNA, was mixed with an equal amount of 70% ethanol in a new tube. RNA was then extracted in the same way as from cell samples but using the RNA mini purification kit by life technologies. RNA was eluted in 80µL nuclease-free water and stored at -80°C, prior to cDNA synthesis.

2.10 cDNA synthesis

cDNA was synthesized from total RNA (500-1000ng) by reverse transcription (RT) using the High Capacity RNA-to-cDNA kit (AppliedBiosystems, ThermoFisher, USA). An RT master mix was made up to contain per reaction; 1µl 20X RT enzyme (MuLV and RNase inhibitor protein), 10µl Buffer (includes dNTPs, random octamers, and oligo dT-16). 11µl of RT master mix was mixed with 9µL of RNA sample in respective wells of a 96 well plate (StarLab, UK) and kept on ice. The plate was then centrifuged, sealed with a StarSeal® aluminum foil cover (StarLab, UK) and placed in a PCR machine - GeneAmp® PCRsystem2700 (AppliedBiosystems, ThermoFisher, USA). The plate went through the following cycle: 60 minutes at 37°C, 5 minutes at 95°C. Samples were then either stored at -20°C or diluted 1:5 in nuclease-free water and used as a template for qPCR.

2.11 Quantitative Polymerase Chain Reaction (qPCR)

The Quantitative Polymerase Chain Reaction technique was used to investigate the expression of multiple genes in this work. qPCR allows for the monitoring of DNA amplification in real time through fluorescence detection. SYBR green is a fluorescent reporter dye which emits when bound to double stranded DNA (binds non-specifically to DNA), unbound SYBR does not fluoresce. In qPCR, fluorescence is measured after every amplification cycle and signal intensity correlates with DNA amount in the sample at that time point. In initial cycles, fluorescence intensity is low and does not exceed background values, but when fluorescence intensity increases above the detectable levels, correlation can be measured. This point is called the quantification cycle. Therefore, if higher DNA concentration is present in a given sample, less amplification cycles will be required for fluorescent intensity to exceed detectable levels.

2.11.1 Primer design for Quantitative Polymerase Chain Reaction (qPCR)

For each gene investigated by qPCR two sets of primers were designed. An 'inner' set of primers were designed for use in the qPCR machine (Thermo Fisher Scientific, United States). An outer set of primers were then designed for creating PCR products to act as standard templates. The inner primers amplify a specific DNA sequence within the gene of interest, whereas the outer primers amplify the region in which the qPCR primers bind. All primers were designed using Primer3 software (available online http://primer3.ut.ee). Primers were designed in accordance with the following specifications (to minimise spontaneity to form primer dimers or hair-pin loops) and can be found in section 2.11.6:

- between 18 and 23 base-pair (bp) in length
- between 40% and 65% GC content (50% optimal)
- Tm of primers within 59.5°C and 61°C (60°C optimal)
- Maximum self-complementarity: 2
- Maximum 3' self-complementarity: 1
- Amplicon size less than 150bp
- Not more than two G or C bases in last 5 at 3' end of each primer (i.e. do not include a GC clamp)
- Avoid stretches of 4 or more G or C bases in a row

If no sequences were suggested by Primer3, the conditions outlined above were gradually relaxed. For example, maximum self-complementarity was relaxed from 2 to 3 and primer Tm down to 59°C. The max 3' self-complementarity condition was not relaxed. BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) was then performed to ensure primer sequences were specific to gene of interest alone. Primers were purchased from Integrated DNA Technologies (Belgium) and were diluted down to a concentration of 0.1nmol/1µl in nuclease free water.

2.11.2 Testing of primer specificity by undertaking standard PCR

All primers were tested for specificity using standard PCR - GeneAmp® PCRsystem2700 (AppliedBiosystems, ThermoFisher, USA) - with a cDNA substrate sourced from resting and activated T-cells and neutrophils. In short, primers were reconstituted in nuclease-free water (0.1nmol/µl) and each primer pair was mixed together. 1µl of the primer mix for the gene of interest, 2µl of the appropriate diluted cDNA, and 2µl of nuclease-free water were added to 45µl of SYBR-green, in a 100µl PCR tube with a cap. The tubes were spun down and the PCR programme proceeded as follows:

1. 3 minutes at 94°C once
2. 40 cycles of:

a) 94°C, 15s
b) 60°C, 10s
c) 72°C, 50s

3. 7 minutes at 72°C

The samples were then kept on ice and loading dye (VWR international, Germany) was added prior to characterisation. These PCR products were characterized by agarose gel (2%) electrophoresis through the use of ethidium bromide (ThermoFisher, USA). 2 grams of Agarose (Scientific Laboratory Supplies, UK) was mixed with 100mL Tris-acetate EDTA buffer and heated until the agarose had dissolved. Ethidium bromide was added to the heated agarose-buffer mix and the gel was poured into a setting tray. The gel was run at 90V in Tris-acetate EDTA buffer for 20 minutes and visualised using ChemiDoc Mp Imaging system (Biorad, Switzerland). PCR primers were deemed suitable for qPCR if single, clear and distinct bands of the correct predicted size were observed. Examples shown in Appendix 1.

2.11.3 Generation of standard curve for qPCR

The generation of a standard curve was essential for the quantification of the expression of genes of interest. In this thesis, absolute level of transcripts was not calculated, as only relative gene expression between samples was of interest. To quantify the relative change in gene expression, the value estimated for each sample was divided by the arbitrary value assigned to the standard. A standard DNA template for each gene assayed was generated by taking the PCR product generated using the "outer" primers, purifying the PCR product on a PCR purification column as per manufacturer's instructions (Qiagen QIAquick columns, UK) and eluting

in nuclease-free water (Qiagen, UK). The eluted, purified product was denoted 10^0 (undiluted), diluted down to 10^{-2} in 10mM TE buffer (enhances stability during freeze/thaw cycles and inhibits DNAses) and stored at -20°C (long-term storage). To generate a standard curve, the 10^{-2} stock was serially diluted from 10^{-4} down to 10^{-9} in nuclease-free water. The wide range allowed genes expressed at very low and very high levels to be quantified, this ensuring that the sample is within detectable range. Furthermore, using a standard curve allows for 1) easier troubleshooting if things go wrong (e.g. contamination); 2) semi-quantification, very useful to know if gene already highly expressed at rest - suggests cells activated in culture.

2.11.4 Quantitative Polymerase Chain Reaction

Each sample and standard were assessed as quadruplicate technical replicates on the qPCR machine - Applied Biosystems quantstudio 7 flex machine (AppliedBiosytems, USA) - in a 384-well plate. A master-mix was prepared for all qPCR reactions (with a 10% excess for pipette errors). For each reaction, the following reagents were used:

- 5µl SYBR green mix (Quanta ROX)
- 0.15µl of primer mix (each primer mixed together at 50 pmol/µl)
- 4µl nuclease free water

4µl of diluted cDNA or standard was placed in a ''mixing'' well (at the bottom), in a 96-well plate. 36µl SYBR-green master-mix generated above was added using a multi-channel pipette and this was mixed by pipetting up and down (for the full 36µl) at least three times. The plate was centrifuged and 9µl of this resulting mix was transferred to each well in a 384-well format using a multi-channel pipette. This approached was taken in order to minimise pipetting errors. A non-template control (NTC) was included to determine the level of contamination and to ensure there was no background signal. The plate was spun for 20 seconds at 400 g to place all contents at the bottom of the well prior to analysis. The plate was sealed with StarSeal Advance Polyolefin seals (Starlab, UK). All product sizes were smaller than 150bp, therefore a fast run was selected on the qPCR machine. The fast run utilises the following reaction conditions:

- Initial 3 minutes at 94°C (not repeated)

- 3 seconds at 94°C then 30 seconds at 60°C repeated 40 times (measuring fluorescence at the end of each 60°C elongation step)

- Melt/ dissociation stage – to measure fluorescence during an incremental increase in temperature from 65°C to 94°C

The melt/dissociation stage was always performed to ensure that the PCR was specific and to confirm that a product of one size only is present.

2.11.5 Normalisation and analysis of qPCR data

The median value of the four replicate reactions, for each biological replicate, was calculated and normalised to a housekeeping gene. Normalisation is necessary to control for any potential differences in absolute quantities of nucleic acid between samples. The housekeeping genes used were either TATA-binding protein (TBP) or GAPDH. Results are expressed as copies of the gene of interest divided by the copies of the housekeeping gene. The quotient was then multiplied by the arbitrary value of $1x10^7$ in order to scale up the values. Analysis of RT-PCR data was done with Microsoft Excel

2.11.6 Primer sequence

2.11.6.1 Human

Table 2.3 Primers

Gene name	Orientation	Sequence	Product size (bp)	NCBI Reference
CXCL8	Forward	agctctgtgtgaaggtgcagt	148	NM_001354840.1
	Reverse	aatttctgtgttggcgcagt		
CXCL8 Standard	Forward	cagagacagcagagcacaca	332	NM_001354840.1
	Reverse	cagttttccttggggtccag		
CCL20	Forward	agcccaagaacagaaagaacc	96	NM_001130046.1
	Reverse	caagtccagtgaggcacaaa		
CCL20 Standard	Forward	gcgaatcagaagcaagcaa	466	NM_001130046.1
	Reverse	agctaaacacagaaaacctacagc		
TNF	Forward	cctgtgaggaggacgaaca	99	NM_000594.3
	Reverse	tgagccagaagaggttgagg		
TNF Standard	Forward	aacctcctctctgccatcaa	502	NM_000594.3
	Reverse	ccaaaggctccctggtct		
TBP	Forward	aggataagagagccacgaacc	137	NM_001172085.1
	Reverse	gctggaaaacccaacttctg		
TBP Standard	Forward	gggcaccactccactgtatc	470	NM_001172085.1
	Reverse	caggaaataactctggctcataact		

2.11.6.2 Mice

Clive, could you kindly put these in for me please, as I do not have the sequences available. I believe we looked at TNF, CCL2, IL-6, CXCL1, CXCL2, and IL-1. Alternatively you could email me the specifications and I can put them in myself. Thanks!

Gene name	Orientation	Sequence	Product size (bp)	NCBI Reference
TNF	Forward	caccaccatcaaggactcaa	96	NM_013693
	Reverse	gaggcaacctgaccactctc		
TNF Standard	Forward	tctgtgaagggaatgggtgt	420	NM_013693
	Reverse	ggctggctctgtgaggaa		
IL-6	Forward	ttccatccagttgccttctt	171	NM_031168
	Reverse	atttccacgatttcccagag		
IL-6 Standard	Forward	tccagaaaccgctatgaagt	370	NM_031168
	Reverse	ctccagaagaccagaggaaa		
CXCL2	Forward	aagtttgccttgaccctgaa	129	NM_009140
	Reverse	tctctttggttcttccgttg		
CXCL2 Standard	Forward	cgcccagacagaagtcatag	484	NM_009140
	Reverse	actcaccctctccccagaaa		
CXCL1	Forward	gcttgccttgaccctgaa	87	NM_008176
	Reverse	tgtcttctttctccgttacttgg		
CXCL1 standard	Forward	ctgggattcacctcaagaaca	469	NM_008176
	Reverse	cttttcgcacaacacccttc		
IL-1	Forward	cgctcagggtcacaagaaac	67	NM_008361.3
	Reverse	gaggcaaggaggaaaacaca		
IL-1 Standard	Forward	aaagtatgggctggactgtttc	410	NM_008361.3
	Reverse	atgtgctggtgcttcattca		
CCL2	Forward	ctcacctgctgctactcattca	153	NM_011333.3
	Reverse	ccattccttcttggggtca		
CCL2 Standard	Forward	caccagcaccagccaact	519	NM_011333.3
	Reverse	gcatcacagtccgagtcaca		

2.12 Luminex

Two 11-plex Luminex kits were purchased from R&D Systems (bio-techne brand), UK. The assay was outsourced and carried out at Wellcome Trust Brenner Building, St James' University Hospital, Leeds.

2.13 Statistical analysis

GraphPad Prism software (USA) was used for data analysis. Non-parametric Mann-Whitney test or parametric student's t-test was used for comparisons between two groups. For comparisons between more than two groups of normally distributed data, ordinary one-way ANOVA test was used. All differences were considered significant at $p<0.05$. All plots have statistical significance indicated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns=not significant.

Chapter 3: Neutrophils and T-cells migratory potential in psoriasis

3.1 Contents

3.2 Introduction and hypotheses

The recruitment of leukocytes and their positioning within the epidermis is a key early event in psoriasis lesion development and is highly dependent on chemokines[798]. Chemokine expression can change rapidly, and this usually reflects the surrounding microenvironment[332-335]. These changes are critical to the cellular composite and leukocyte positioning[332-335]. Although some of the biologic therapies (for the purposes of this thesis the term 'biologic' will refer to monoclonal antibodies) available appear to profoundly impact leukocyte positioning in skin, an effective leukocyte chemotaxis targeting agent is yet to be developed[725]. Rather, the majority of the approved drugs for the treatment of psoriasis target immune cell function non-specifically, or target selected pro-inflammatory cytokines (e.g. TNF, IL-23, IL-17). However, new evidence suggests that the targeting of different immune pathways, such as immune cell migration, might improve treatment specificity[798,853]. Therefore, investigating the role of psoriasis-related chemokines and their adjacent receptors in leukocyte recruitment to the epidermis is critical for the identification of new therapeutic targets.

Researching leukocyte migration could allow for the development of novel treatments that target epidermal leukocyte infiltration more specifically and therefore alleviate plaque development. Previous work has demonstrated elevated levels of both neutrophils, T-cells and chemokines, such as CXCL8, CCL2, CCL5, CCL17, CCL18, CCL27, CXCL10, CXCL16 and CX3CL1, in the psoriatic lesion (Table 2)[2,760,830]. It has, however, never been shown if the aforementioned chemokines can attract leukocytes in the context of psoriasis. In this chapter, **we hypothesised that chemokines, expressed highly in psoriatic lesions, have the ability to attract neutrophils and T-cells from the peripheral blood of patients with psoriasis.** Importantly, while some chemokine/receptor pairs are known to be important for cell migration to specific tissues, others do not show such associations[830,831]. Therefore, **it was further hypothesised that not all psoriasis-associated chemokines will exert the same chemoattractive properties.** The increase in neutrophils in the plaques can be explained by a concomitant increase of neutrophil chemotactic factors and neutrophil peripheral blood levels[897]. However, it has been shown, as discussed in Chapter 1, that psoriasis involves a systemic increase of pro-inflammatory mediators[2]. This could conceivably induce the stimulation and activation of neutrophil migration prior to their arrival at the plaques. Therefore, **we also hypothesised that the increase of neutrophil numbers in the plaque is due to the cells being primed for migration in the periphery, rather than simply reflecting their increase in blood.** Although, current drugs for the treatment of psoriasis do not target leukocyte migration directly, they are associated with

a reduction of leukocyte numbers in the lesions[725]. **Therefore, we hypothesised that commonly utilised drugs for the treatment of psoriasis might have an effect on leukocyte migration.**

In order to study these hypotheses, transwell permeable supports (TPS) were utilised. TPS are designed to produce a cell culture environment closely resembling the *in vivo* state, allowing us to develop a psoriasis-specific transmigration assay. TPS is a well-established *ex vivo* system, which can be optimised in accordance to many different conditions (e.g. cell types and their origin, chemokines of interest) and diseases. To achieve a psoriasis-like setting, we selected chemoattractants that are known to be elevated in the psoriasis lesions and isolated leukocytes from the peripheral blood of patients with psoriasis. We reasoned that leukocytes responsive to chemokines elevated at the psoriatic lesions, would be more likely to be recruited to the plaques *in vivo*. Further to this, chemokine concentrations were optimised; membrane pore sizes were selected in accordance to cell diameter, in an attempt to resemble *in vivo* transmigration. The assay allowed for the investigation of leukocyte migration and the role of key psoriasis-related chemokines in an *ex vivo* system. Further to this, the assay became a useful tool for the selection of leukocytes, which we hypothesised would be more prone to infiltrate the inflammatory lesions, from the peripheral blood of patients with psoriasis.

Thus, the newly devolved *ex vivo* psoriasis-like model for studying leukocyte migration, more closely mimics *in vivo* migration to and within the lesion. This allowed us to investigate the functional relevance of chemokines and assess the migratory capacity of leukocytes in the context of psoriasis. Furthermore, the assay allowed us to compare the migratory potential of leukocytes derived from patients with psoriasis and other inflammatory conditions, such as neutrophilic dermatoses and psoriatic arthritis.

3.3 Chapter aims

- Optimise a migration assay, which would allow for the selection of leukocytes fromperipheral blood, on the basis of their responsiveness to chemokines elevated at the psoriatic lesion
- To study leukocyte migration and identify chemokines key to the recruitment of leukocytes to psoriasis lesions
- To define the differences between the migratory potential of healthy donor-derived and patients with psoriasis-derived leukocytes
- To assess the effect of different drugs for the treatment of psoriasis on leukocyte migration

3.4 Results

3.4.1 Optimisation of transwell assay and the impact of psoriasis-related chemokines on leukocyte recruitment

The migration of leukocytes is controlled by a complex and dynamic chemokine network. In the context of psoriasis, inflammatory cells are highly enriched in the lesions which is paralleled by the elevated levels of chemokine expression. Previous work has described multiple psoriasis-related chemokines in psoriatic skin, but the functional relevance of the majority of them remains unknown. Where transmigration studies have been conducted, chemokine concentrations used vary greatly or/and are too high to be considered physiologically relevant[837]. Here, we optimised chemokines used and their concentration for the investigation of neutrophil and T-cell transmigration. Further to this, we demonstrated the ability of psoriasis-associated chemokines to attract leukocytes.

To develop the leukocyte transmigration assay, neutrophils utilised were derived from the blood of patients with psoriasis or non-psoriasis control donors. In this thesis, the terms 'psoriatic T-cells/neutrophils' and 'healthy T-cells/neutrophils' refer to cells which have been derived from patients who have psoriasis or from non-psoriasis donors, respectively. The initial stages of this study were presented with certain limitation, such as the inability to recruit patients with psoriasis for an interval of time, due to a departmental MHRA inspection. Therefore, during this time period we diversified our experimental methodologies and e.g. used resting and activated healthy donor-derived T-cells in the transwell optimisation stages. We reasoned that the phenotype of psoriatic T-cells would resemble this of activated healthy T-cells. This was later repeated using T-cells from patients with psoriasis. To be able to define the differences in migratory potential between healthy donor- and psoriasis-derived leukocytes, we optimised the conditions as outlined below. For the purposes of this thesis the term 'psoriasis; psoriasis-derived' will only refer to psoriasis, and will not encompass psoriatic arthritis)

To develop the assay and identify chemokines key to the recruitment of leukocytes to psoriasis lesions we created a panel of chemokines. Chemokines used were selected based on previous reports of elevated levels in psoriasis lesions. We also took into account previous data of leukocyte receptor expression, for the chemokines of interest, under inflammatory conditions. For example, neutrophils are known to be highly responsive to CXCL8[898]; however, they have also

been shown to upregulate the expression of various chemokine receptors, such as CCR2 and CXCR6, in the context of inflammation[836,899]. Based on these criteria CXCL8, CXCL16 and CCL2 were selected for the neutrophil transmigration assays (Figure 3.1); and CCL5, CCL18, CXCL10, CCL20, CCL17, CCL27, CXCL16, CX3CL1 for the T-cell transmigration assay (Figure 3.2). To determine an optimal chemokine concentration, a range of concentrations for each chemokine of choice was tested; 10ng/mL, 50ng/mL, 200ng/mL, and 500ng/mL, based on previously published data[837,900,901]. The timing of migration was also optimised. It was determined that 1h incubation period was best suited for neutrophil migration (data shown in the appendix A) and 1.5h for T-cells (as per previously published data)[900-902].

Neutrophils derived from healthy and psoriatic donors did not exhibit a significant increase in migration rate towards CCL2 or CXCL16 (Figure 3.1 A and B). Similarly, 10ng/mL of CXCL8 was not potently attract healthy donor-derived neutrophil (Figure 3.1 A). In contrast, healthy donor-derived neutrophils exhibited a significant increase in migratory response to 50ng/mL and 200ng/mL of CXCL8 (33.1% and 36.3% of total neutrophils, respectively) (Figure 3.1 A). In comparison, psoriatic neutrophils showed significant chemoattraction to all CXCL8 concentrations tested (Figure 3.1 B). Here we report that the rate of migration towards all concentrations was similar: 10ng/mL induced 52% of total patient-derived neutrophils to migrate; 50ng/mL – 57.8%; and 200ng/mL – 55.6% (Figure 3.1 B).

When selecting the most appropriate chemokine and chemokine concentration for further experiments, chemokine's ability to induce chemoattraction, chemokine maximisation and reduction of unspecific attraction were taken into account. Therefore, to assess neutrophil migratory potential and select neutrophils from the peripheral blood of healthy and psoriatic donors, 50ng/mL of CXCL8 was selected. The use of CCL2 and CXCL16 was discontinued in future experiments, due to the insignificant neutrophil attraction they induced at this stage.

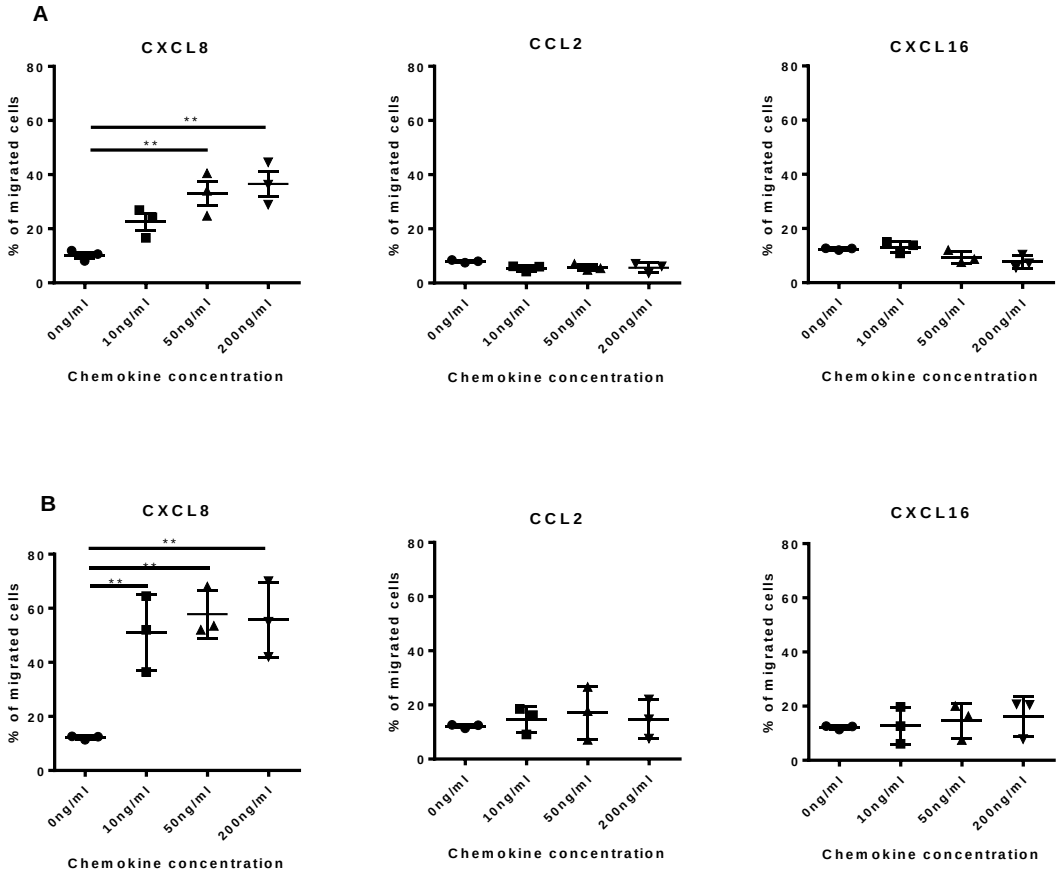


Figure 3.1| Optimisation of chemokines and chemokine concentration for the transmigration analysis of neutrophils derived from healthy or psoriatic donors

*Optimisation of the migration of neutrophils, expressed as percentage of total neutrophils, derived from (A) healthy or (B) psoriatic donors, in response to rising concentrations of CXCL8, CCL2, and CXCL16. Following a 1h incubation period in transwell chambers (6.5mm, 3μM pores), migrated cells were collected and counted. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (n=3 samples) (One-way ANOVA test; **p<0.01).*

As mentioned earlier, the early stages of the study were hampered by a shortage of patients available for recruitment due to factors outwith the university's control. As a result, the T-cell chemokine optimisations were completed using T-cells from healthy donors, which were then activated. This was later repeated with T-cells from patients with psoriasis. CCL18, CCL27, and CX3CL1 did not induce a significant increase in migration of non-activated and activated healthy donor-derived T-cells (Figure 3.2 A and B). 200ng/mL of CCL5 was potent at attracting resting, but not activated T-cells derived from healthy donors. In contrast, CCL17 (both concentrations tested) induced a significant increase in migration of activated, but not resting T-cells. CXCL10 and CCL20 were the only chemokines to attract both resting (at 200ng/mL) and activated T-cells (50 and 200 ng/mL) at a significant rate. CCL5, CXCL10 and CCL20 were later confirmed to be able to attract psoriatic T-cells (Figure 3.3). Due to the data presented here and the role of CCL20 in the pathogenesis of psoriasis (IL-23/Th17 axis), CCL20 was selected for the studying of T-cell migratory potential and T-cell selection from peripheral blood.

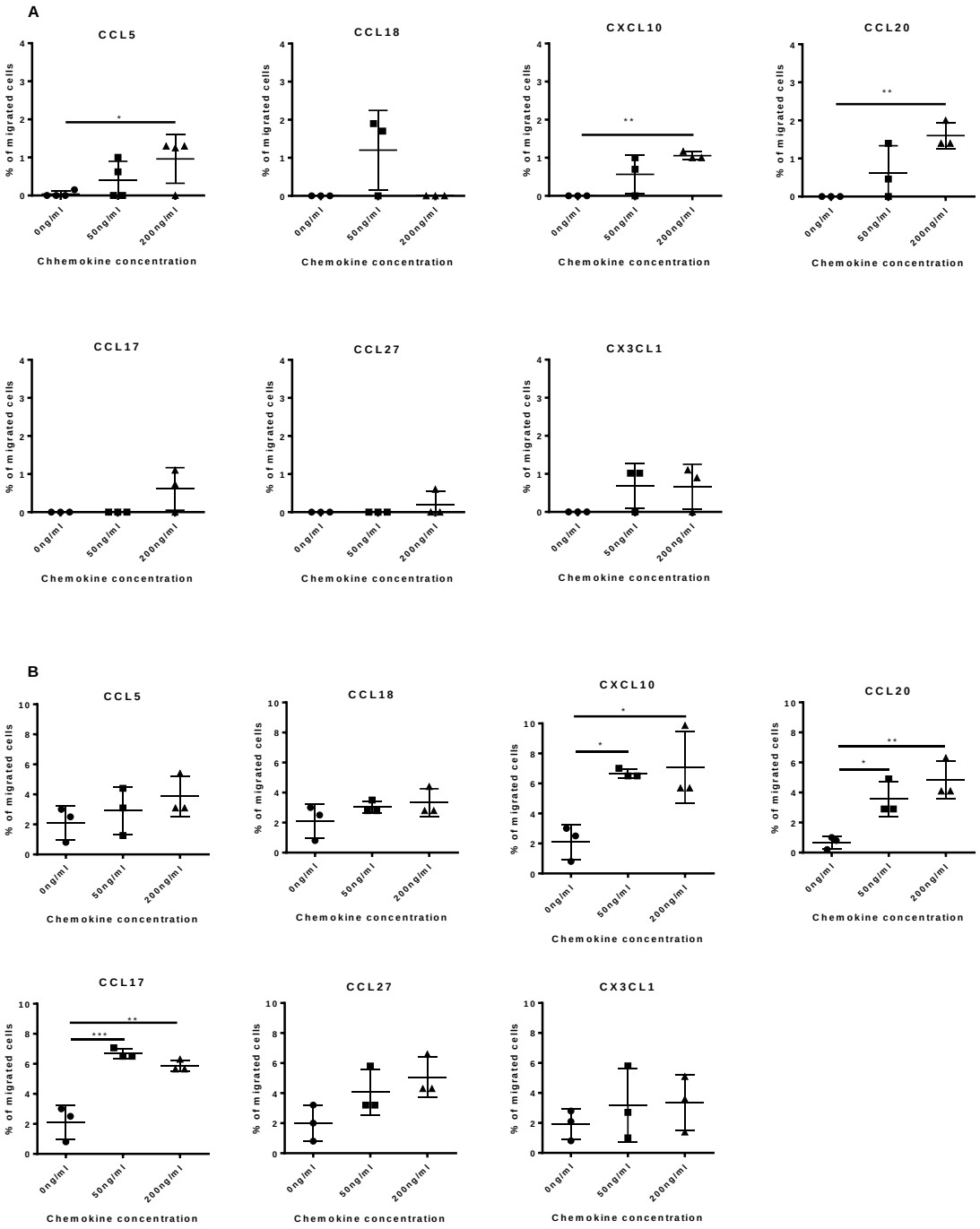


Figure 3.2| Optimisation of chemokines and chemokine concentration for the transmigration analysis of resting or activated T-cells derived from healthy donors

*Optimisation of the migration of resting (A) or CD3/CD28-activated (B) T-cells, expressed as percentage of total T-cells, derived from healthy donors, in response to rising concentrations of CCL5, CCL18, CXCL10, CCL20, CCL17, CCL27, and CX3CL. Following a 1.5h incubation period in transwell chambers (6.5mm,5μM pores), migrated cells were collected and counted. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (n=3 samples) (One-way ANOVA test; *p<0.05; **p<0.01; ***p<0.001).*

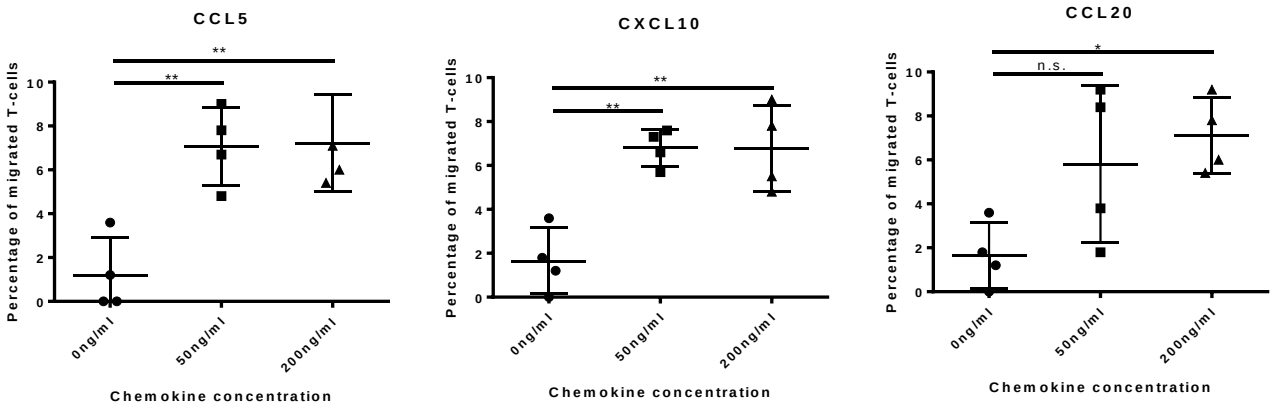


Figure 3.3| Optimisation of chemokines and chemokine concentration for the transmigration analysis of T-cells derived from patients with psoriasis

*Optimisation of the migration of T-cells, expressed as percentage of total T-cells, derived from patients with psoriasis, in response to rising concentrations of CCL5, CXCL10, and CCL20. Following a 1.5h incubation period in transwell chambers (6.5mm,5 μM pores), migrated cells were collected and counted. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (n=4 samples) (One-way ANOVA test; n.s. = non-significant *p<0.05; **p<0.01).*

3.4.2 Neutrophils and T-cells derived from patients with psoriasis have an enhanced migratory potential

After identifying what chemokines were able to recruit leukocytes in the context of psoriasis, we wanted to determine what factors influenced leukocyte plaque infiltration. We first determined the number of leukocytes in the peripheral blood of patients, as an increase in the plaques could be a reflection of an increase in the periphery. Neutrophil and T-cell numbers in the peripheral blood of patients with psoriasis increased by 2- and 1.8-fold, respectively, when compared to healthy donor's leukocyte numbers (Figure 3.4).

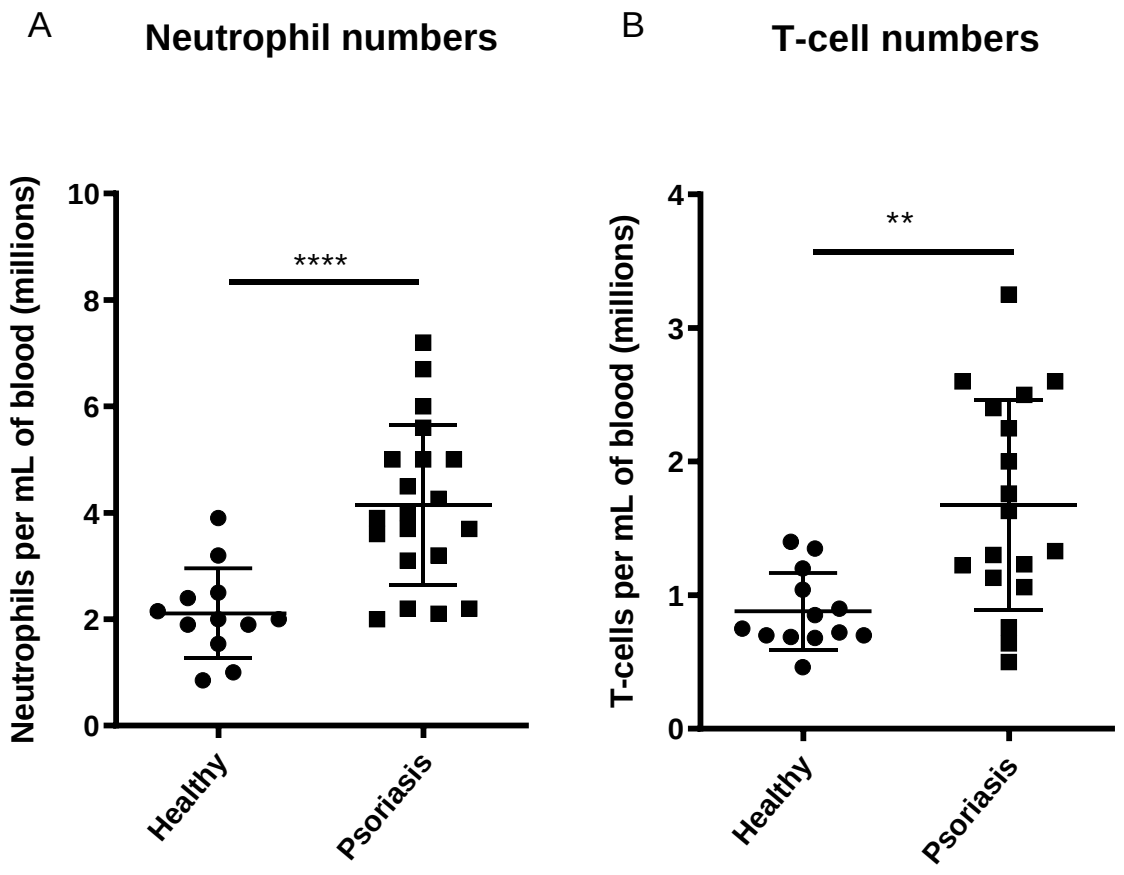


Figure 3.4| Patients with psoriasis had higher levels of neutrophils and T-cells per mL of blood than healthy donors

*Neutrophils (A) and T-cells (B) were isolated from the blood of healthy or psoriatic donors and counted. Counts are represented as Neurtophils/T-cells per mL of blood. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (A: n=12 healthy and 18 psoriatic samples; B: 12 healthy and 18 psoriasis samples) (Mann-Whitney test; **p=0.0026; ****p<0.0001).*

The finding that leukocyte numbers are elevated in the blood of patients with psoriasis prompted us to determine if the increase of leukocyte numbers in the plaques was influenced by cells' migratory potential; rather than just be a consequence of elevated peripheral blood leukocyte numbers and plaque chemokine levels. To investigate this, neutrophils and T-cells from psoriatic and healthy donors were migrated towards CXCL8 and CCL20, respectively. The decision to use CXCL8 and CCL20 was based on the findings described in sub-chapter 3.4. Cells derived from healthy controls were utilised, whilst cells from patients with neutrophilic dermatoses (ND) and Psoriatic arthritis (PsA) were used as comparators, to determine whether any differences were specific to psoriasis, or a generalisable phenomenon in inflammatory disease, including the key conditions ND and PsA.

Neutrophils and T-cells derived from patients with psoriasis (mild to severe disease patients included), exhibited a significantly greater migratory capacity, when compared to neutrophils and T-cells derived from healthy donors. Responsiveness to CXCL8 and CCL20 of neutrophils and T-cells, derived from patients with psoriasis, increased by 2- and 2.9-fold, respectively, when compared to their healthy counterparts (Figure 3.5 & 3.6). In contrast, neutrophils, but not T-cells, derived from patients with NDs or PsA exhibited an increase in CXCL8 responsiveness, but not to the same degree as psoriasis-derived samples. Neutrophils and T-cells from healthy donors did not exhibit a significant migratory increase when compared to cells derived from the other groups. This suggested that the increase of leukocytes in the plaques is not merely due to an increase in chemokine levels and peripheral leukocyte numbers, but due to changes in migratory potential/chemokine responsiveness.

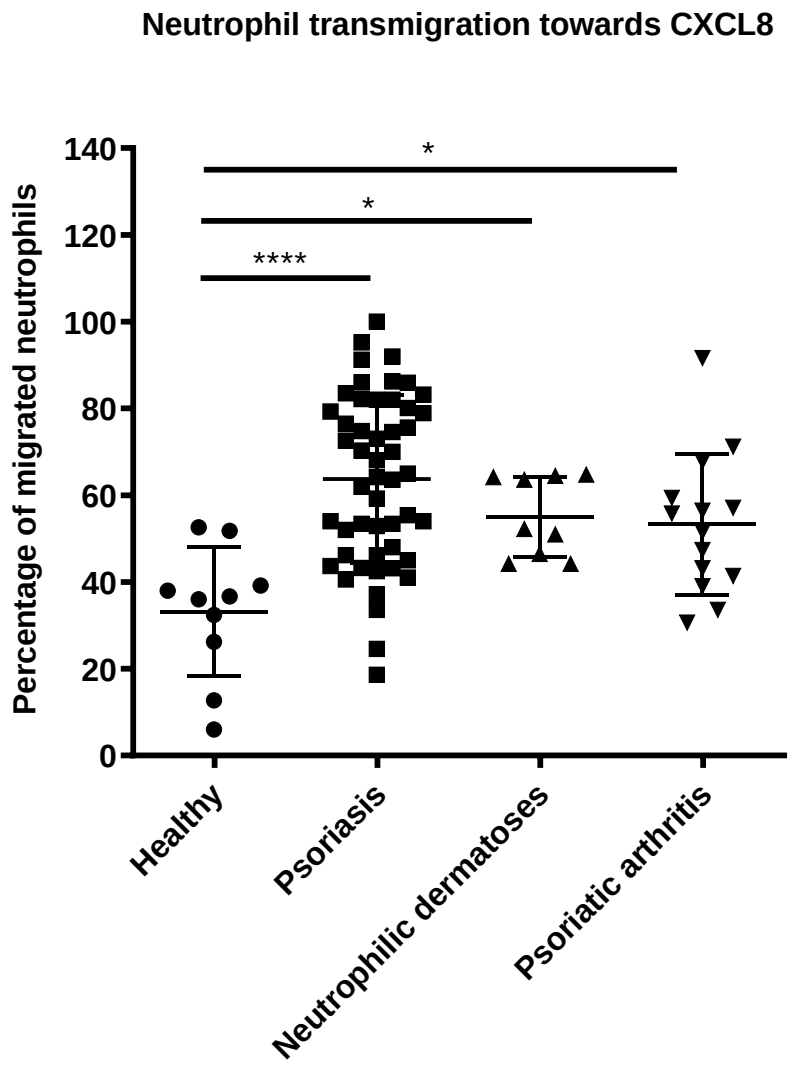


Figure 3.5| Neutrophils derived from patients with psoriasis, neutrophilic dermatoses, and psoriatic arthritis were more migratory towards CXCL8 than healthy donor-derived neutrophils

*Transwell analysis of the migratory capacity of neutrophils derived from healthy donors, patients with psoriasis, patients with neutrophilic dermatoses (NDs), and patients with psoriatic arthritis (PsA). Migrated cells are expressed as percentage of total neutrophils that were placed on the membrane. Migration was induced by 50ng/ml CXCL8. Following a 1h incubation period in transwell chambers (6.5mm, 3 μM pores), migrated cells were collected and counted. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (12 healthy; 50 psoriasis; 9 NDs; 14 PsA samples) (one-way ANOVA test; ****p<0.0001; *p=0.0237; *p=0.0202).*

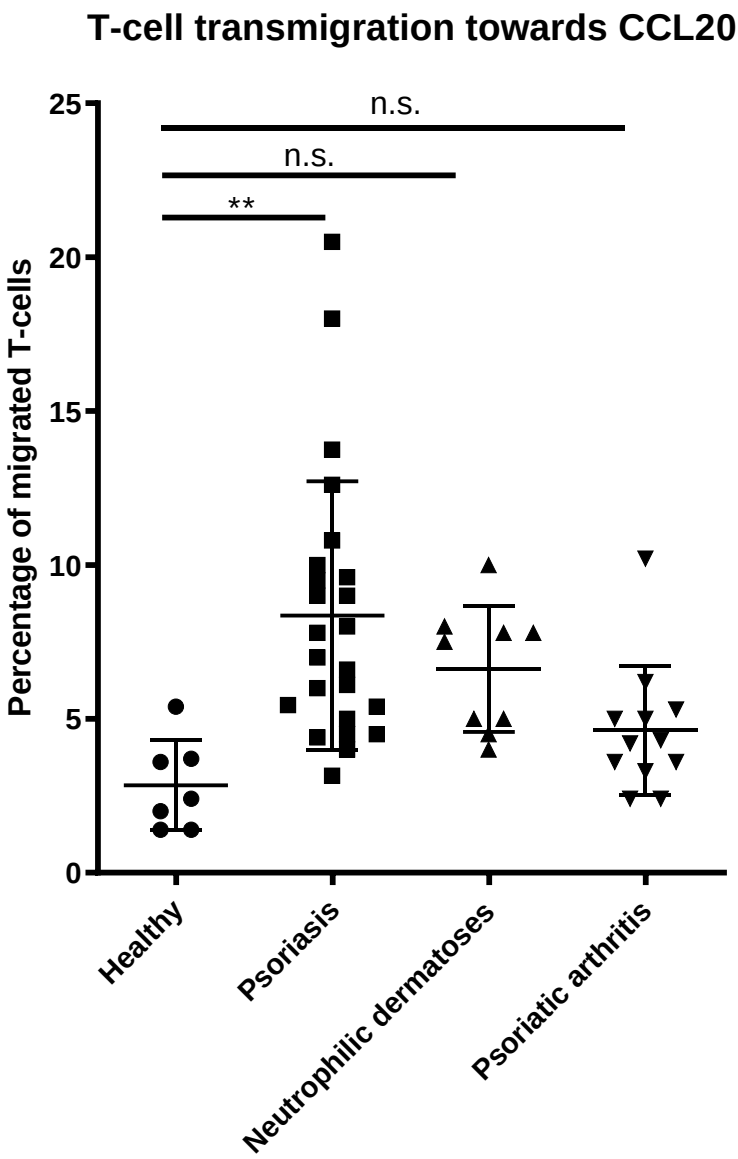


Figure 3.6| T-cells derived from patients with psoriasis were more migratory towards CCL20 than healthy donor-derived T-cells.

Transwell analysis of the migratory capacity of T-cells derived from healthy donors, patients with psoriasis, and patients with neutrophilic dermatoses. Migrated cells are expressed as percentage of total T-cells that were placed on the membrane. Migration was induced by 200ng/ml CCL20. Following a 1.5h incubation period in transwell chambers (6.5mm, 5 μM pores), migrated cells were collected and counted. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (7 healthy; 24 psoriasis; 9 NDs; 12 PsA samples) (one-way ANOVA test; p=0.0010). n.s. – non significant

Interestingly, the increase in CXCL8-responsiveness by psoriatic neutrophils was not associated with higher CXCR2 (CXCL8 receptor) cell surface expression levels (Figure 3.7 & 3.8). Although this finding would suggest that other mechanisms are responsible for this heightened responsiveness, it should be noted that CXCR2 is not the only CXCL8 receptor. Indeed, the chemokine/chemokine receptor system is very promiscuous, and one ligand can bind multiple receptors and induce a similar outcome. Therefore, the expression of different to CXCR2 receptors, which can bind CXCL8, should be examined to better understand the mechanisms responsible for the increase in responsiveness.

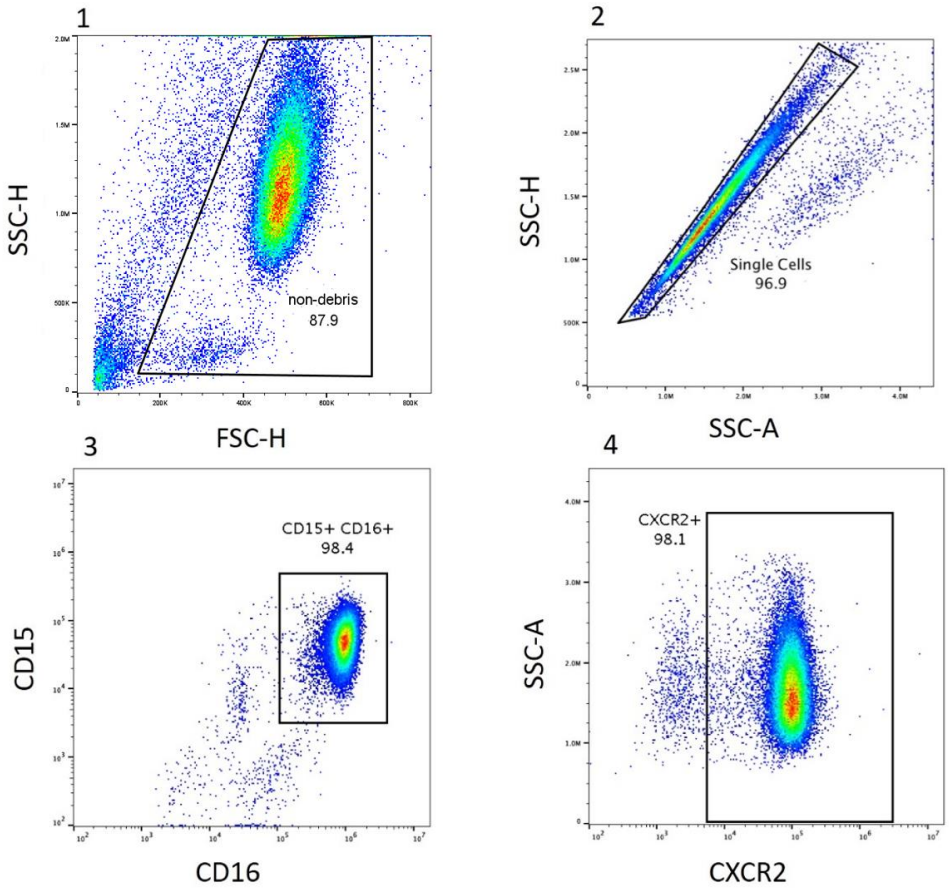


Figure 3.7| Flow-cytometric gating strategy for the identification of CXCR2+ neutrophils from the peripheral blood of humans.

Neutrophils were isolated using the EasySep StemCell kit for the direct isolation of neutrophils from peripheral blood. The isolated cells were stained with antibodies against CD14, CD15, and CXCR6, within an hour of isolation. Events were gated on forward and side scatter and debris were excluded (1); doublet cells were then excluded by gating side scatter height vs area (2); neutrophils were then identified on the basis of CD15 and CD16 (3) and gated for CXCR2 expression (4). This gating strategy was carried out on psoriatic neutrophils (n=4).

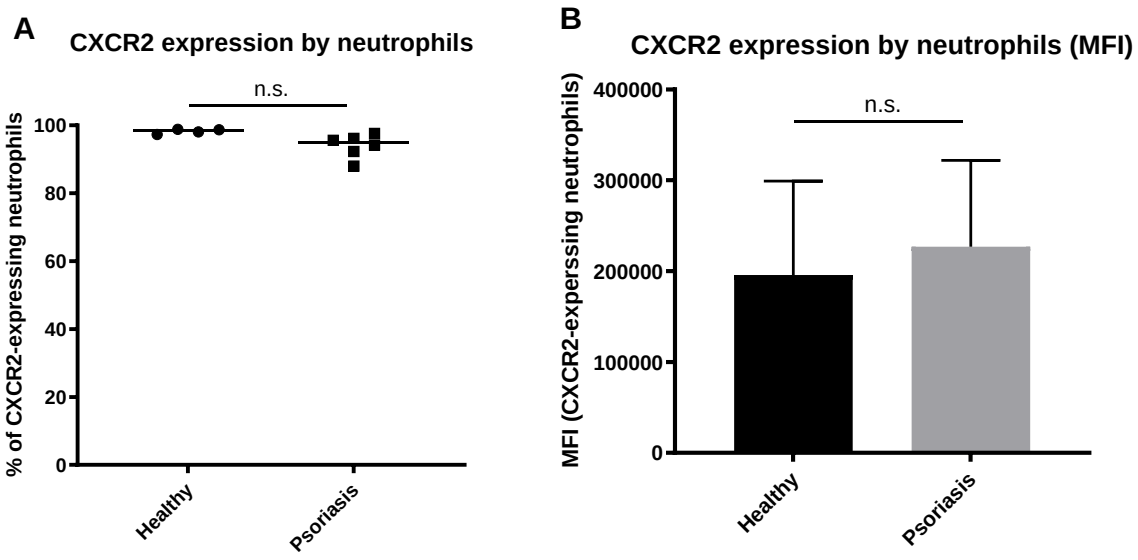


Figure 3.8| Neutrophils derived from healthy donors and patients with psoriasis had a similar cell surface expression of CXCR2

Flow-cytometric analysis of the CXCR2 surface expression of neutrophils derived from healthy donors or patients with psoriasis. Neutrophils were isolated from peripheral blood and stained with antibodies against CD15, CD16 and CXCR2. Expression of CXCR2 is presented as (A) percentage of CXCR2-expressing CD15+CD16+ cells from total CD15+CD16+ cells in each sample or (B) mean fluorescence intensity of CXCR2-expressing cells. Data in (A) are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD; Data in (B) are presented as bar graphs with Mean+SD (4 healthy; 6 psoriatic samples) (Unpaired t-test); n.s. = non-significant

3.4.3 Determining the relationship between migration and PASI/age/sex of patients

Following the confirmation that the increase of leukocyte numbers in the psoriasis plaques is influenced by cells' migratory capacity, we wanted to determine the relationship between patients' leukocyte migration and key clinical characteristics. This could reveal if neutrophil migration and therefore plaque inflammation severity is dependent on age or sex. Further to this, we wanted to establish if PASI scores can predict heightened leukocyte migration. A correlation between neutrophil and T-cell (Figure 3.9 A) migration and patients PASI score was not found. Leukocyte migration did not correlate with patients' age either (Figure 3.9 B). Further to this, there was not a significant difference between neutrophil (Figure 3.10 A) and T-cell (Figure 3.10 B) migration of male and female patients. Based on the clinical characteristics examined in these experiments, leukocyte migration rates were unaffected by age, sex and clinical psoriasis severity as assessed by the PASI.

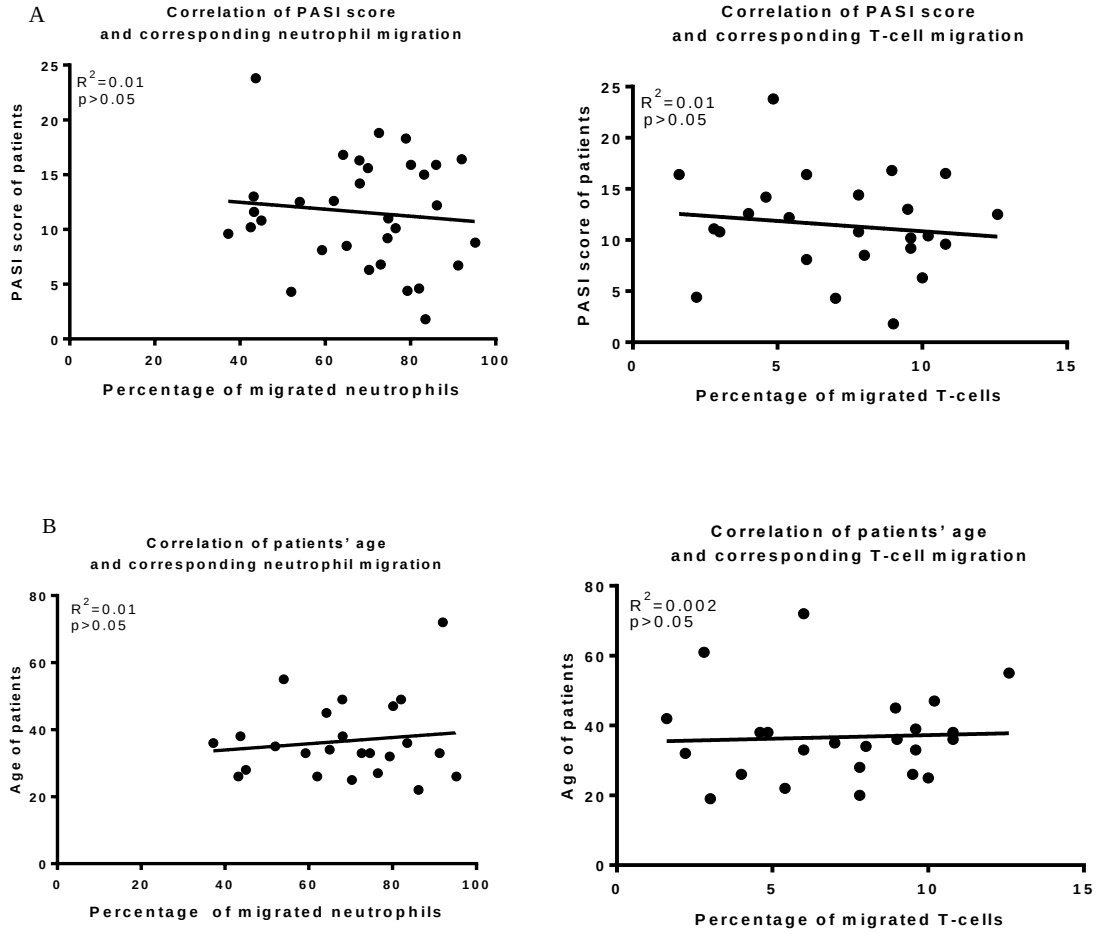


Figure 3.9| Migration of neutrophils and T-cells derived from patients with psoriasis did not correlate with corresponding PASI scores or age

Relationship between (A) PASI score or (B) age of patients with psoriasis and corresponding neutrophil (left) or T-cell (right) migration values. Each dot represents (A) PASI or (A) age x migration of a particular patient. Statistical analysis is nonlinear fit (correlation). R^2=0.01; p>0.05

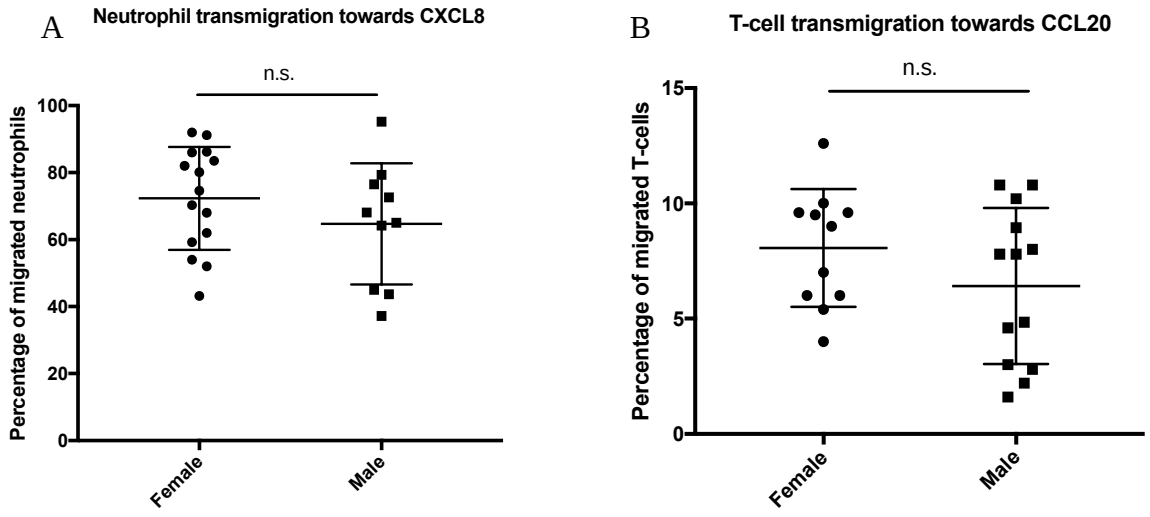


Figure 3.10| The responsiveness of patients with psoriasis-derived neutrophils and T-cells to CXCL8 and CCL20, respectively, was not related to sex

Transwell analysis of the migratory capacity of neutrophils (A) and T-cells (B) derived from patients with psoriasis. Migrated cells are expressed as percentage of total neutrophils or T-cells that were placed on the membrane. Migration was induced by 50 ng/mL CXCL8 or 200 ng/mL CCL20. Following a 1h or 1.5h incubation period in transwell chambers (6.5mm, 3 µM or 5 µM pores), migrated cells were collected and counted. . Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (A: 25 patient samples (15 female and 10 male) B: 24 patient samples (11 female and 13 male)) (Mann-Whitney test; A: p=0.3110; B: p=0.2699; n.s. = non-significant)

3.4.4 The effect of different drugs for the treatment of psoriasis and psoriatic arthritis on neutrophil and T-cell transmigration

The findings described in earlier sections suggested that, in psoriasis, neutrophils and T-cells exhibit an enhancement of their migratory potential, which could result in more leukocytes infiltrating the plaques. These data are also included in the figure below (3.11) as a comparator for the other treatment groups. Upon treatment of patients with psoriasis with anti-inflammatory drugs, such as Apremilast, Secukinumab and anti-IL-23 biologics, leukocyte numbers in plaques would be expected to be reduced[88a,725]. Therefore, we wanted to investigate the possibility that such treatments might affect leukocyte migration towards chemotactic cues. Neutrophils and T-cells were isolated from patients treated with Apremilast, Secukinumab and anti-IL-23 biologics and were migrated towards CXCL8 and CCL20, respectively. Neutrophils derived from patients treated with Apremilast, but no other drug, had a reduction of their migratory potential (Figure 3.11 A). T cell migration was not affected by the treatment of patients with any of the drugs taken into account in this study (Figure 3.11 B). Apremilast is a PDE4 inhibitor and therefore has a very broad effect in terms of affected cytokine pathways. These data suggest that a possible mechanism of action of Apremilast is the direct reduction of neutrophil migration. Therefore, we decided to further explore the effect of Apremilast on neutrophil migration in the next chapter. In addition to psoriasis, we also investigated the effects of anti-inflammatory drug methotrexate on the migration of neutrophils derived from patients with psoriatic arthritis. Our findings indicated that methotrexate did not reduce CXCL8-responsiveness of neutrophils derived from patients with psoriatic arthritis (Figure 3.12)

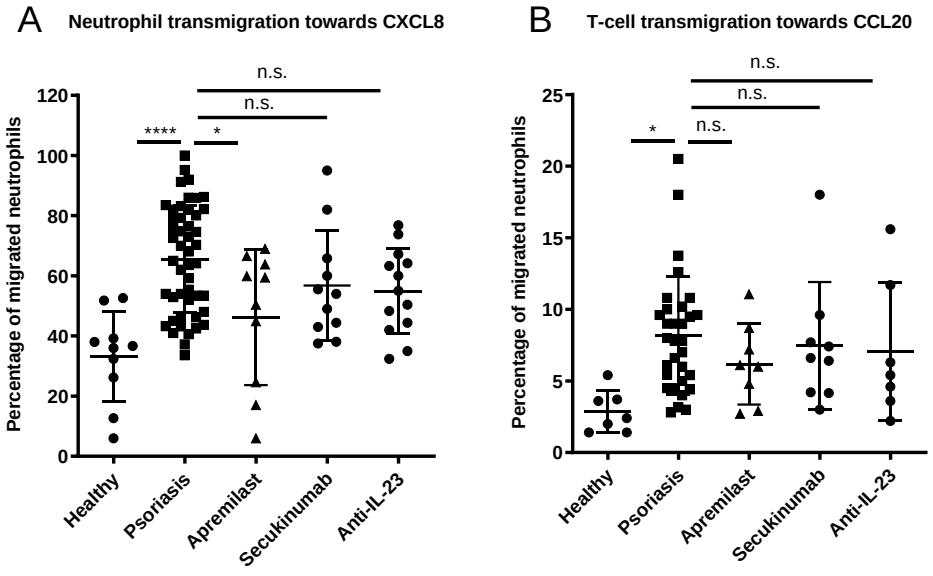


Figure 3.11| The effect of different drugs for the treatment of psoriasis on neutrophil and T-cell transmigration

*Transwell analysis of the migratory capacity of neutrophils (A) and T-cells (B) derived from healthy donors, patients with psoriasis, and patients with psoriasis treated with Apremilast, Secukinumab or anti-IL-23 agents. anti-IL-23 agents include Guselkumab, Ustekinumab, Risankizumab. Migrated cells are expressed as percentage of total neutrophils or T-cells that were placed on the membrane. Migration was induced by 50ng/ml CXCL8 (A) or 200ng/mL CCL20 (B). Following a 1h (A) or 1.5h (B) incubation period in transwell chambers (6.5mm, 3 μM pores (A) or 5 μM pores (B)), migrated cells were collected and counted. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (A: 10 healthy, 50 psoriasis (untreated), 10 Apremilast, 11 Secukinumab, 13 anti-IL-23 samples; B: 7 healthy, 31 psoriasis (untreated), 8 Apremilast, 9 Secukinumab, 7 anti-IL-23 samples) (one-way ANOVA test; A: *p=0.0188; ****p<0.0001; B: *p=0.0154) n.s. = non-significant.*

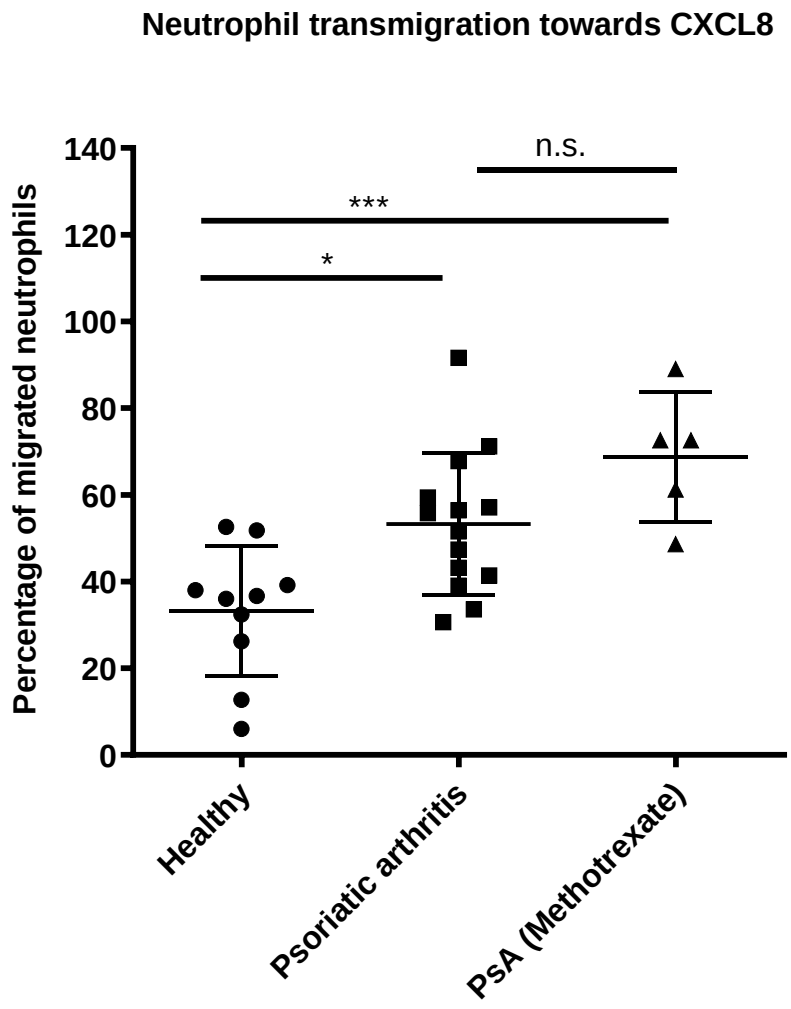


Figure 3.12|The effect of methotrexate treatment on patients with PsA-derived neutrophil transmigration

*Transwell analysis of the migratory capacity of neutrophils derived from healthy donors, patients with psoriatic arthritis, and patients with psoriatic arthritis treated with Methotrexate. Migrated cells are expressed as percentage of total neutrophils or T-cells that were placed on the membrane. Migration was induced by 50ng/ml CXCL8. Following a 1h incubation period in transwell chambers (6.5mm, 3 μM pores), migrated cells were collected and counted. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (A: 10 healthy, 14 patients with psoriatic arthritis, 5 Methotrexate) (one-way ANOVA test; A: *p=0.0188; ***p<0.001) n.s. = non-significant.*

3.5 Summary and conclusions

In this initial chapter, we hypothesised that chemokines, expressed highly in psoriatic lesions, have the ability to attract neutrophils and T-cells from the peripheral blood of patients with psoriasis; and that leukocyte migratory potential might be enhanced. A plethora of chemokines are expressed at high levels in the psoriasis lesion and leukocyte numbers are elevated[2,760,830,897]. It is not clear if and what psoriasis-associated chemokines can attract leukocytes to the lesions. It is also unclear if elevated neutrophil levels in the plaques mirror the increase in peripheral blood, or their migratory potential is enhanced. To investigate this, we adapted the transwell assay to resemble key aspects of the psoriatic setting. This was achieved by making use of psoriatic leukocytes and psoriasis-associated chemokines. Previous studies have also utilised the transwell permeable supports to study cell migration[837]; however, very few such studies have been carried out using psoriatic leukocytes and often used too high chemokine concentrations[837]. The *ex vivo* transmigration assay allowed us to evaluate the chemoattractive potential of selected psoriasis-associated chemokines; select the most-appropriate chemokine to study leukocyte migratory potential; study leukocyte migratory potential and select migration-prone leukocytes from peripheral blood.

In the first set of experiments our aims were to examine the ability of psoriasis-associated chemokine to attract neutrophils and T-cells derived from patients with psoriasis. This would also allow us to choose the most appropriate psoriasis-associated chemokine to test leukocyte migratory potential with; and subsequently select leukocytes that are more likely to migrate to the lesions, from peripheral blood. All chemokines were selected based on expression elevations in the lesions and reports of leukocyte receptor expression under inflammatory conditions.

We demonstrated that CCL2 and CXCL16 do not attract neutrophils derived from patients with psoriasis or healthy donors in our set-up. Due to the inability of CCL2 and CXCL16 to attract neutrophils, at even as high concentration as 200ng/mL, they were ruled out as possible molecules to be used in assessing neutrophil migratory potential and selection from peripheral blood. Although these data suggest that CCL2 and CXCL16 might not play a crucial role in neutrophil recruitment to psoriasis lesions, Steffen *et al*. demonstrated that CXCL16 was able to induce neutrophil migration in the context of psoriasis[837]. It is important to note; however, that the concentrations of CXCL16 used in those studies were particularly high, which might undermine

the physiological relevance of their data. Interestingly, CXCL16 was shown to play an important role in enhancing CXCL8-induced neutrophil migration, by the same group. This suggests that chemokines in the context of psoriasis might act co-operatively to induce the inflammatory response. Although it would have been interesting to examine the combined effects of the chemokines of interest on neutrophil migration, our study aims were to decipher the individual impact of each chemoattractant.

In contrast to CCL2 and CXCL16, CXCL8 was able to induce significant migration of neutrophils. It is important to highlight that the neutrophils from each donor were migrated against CCL2, CXCL16, and CXCL8 under identical conditions. This confirms that the lack of migration against CCL2 and CXCL16 did not stem from a technical issue, but rather it likely related to the biology of the cells. Interestingly, only medium- to high CXCL8 concentrations (50-200ng/mL) could attract healthy donor-derived neutrophils, whereas patients with psoriasis-derived neutrophils were also attracted by low CXCL8 concentrations (10ng/mL). This suggested that the migratory potential of psoriatic neutrophils could be altered. It is important to mention that a full dose-response curve for CXCL8 responsiveness was not generated. Generating such a curve would have better informed the decision of what CXCL8 (and CCL20) concentration to use in the chemokine responsiveness experiments, discussed below. Crucially, psoriasis is a systemic condition, associated with an increase in pro-inflammatory cytokine levels not only in the lesions, but also the periphery[2]. The systemic increase of pro-inflammatory factors could therefore reasonably be expected to affect circulating leukocyte function. Early studies demonstrated that neutrophil chemotactic activity could be enhanced by preincubation with human plasma from patients with extensive psoriasis lesions; however, this was not the case when the plasma was derived from healthy donors[896]. These data are in support of our findings and hypotheses and suggest that neutrophils in psoriasis might have an increase in sensitivity to CXCL8. In addition, our data also showed that leukocyte numbers are elevated in the peripheral blood of patients with psoriasis. Considering these findings, we went on to determine if the increase in lesional neutrophil numbers is due to the cells being primed for migration in the periphery, rather than merely reflecting their increase in blood. Here we report that psoriatic neutrophils have an enhanced responsiveness to CXCL8, when compared to neutrophils derived from healthy donors. Interestingly, the increase in responsiveness could not be attributed to an increase in CXCR2 expression on neutrophils. However, it is important to note that CXCL8 can bind to more than one receptor (e.g. CXCR1) and induce migration. Therefore, it would be useful to examine the expression of all CXCL8 receptors on neutrophils derived from patients with psoriasis. Furthermore, pro-inflammatory stimuli could act on different intracellular pathways responsible

for the induction of migration. Importantly, our findings suggest that neutrophil migratory potential might be affected in the periphery and are in unison with the early studies discussed above.

We then focused on investigating T-cell migration. At this stage of the study, patient recruitment was discontinued for a limited amount of time. This necessitated a change in strategy – T-cells were isolated from healthy donors and activated via CD2/CD3/CD28 stimulation in place of blood samples from psoriasis donors. CCL18, CCL27, and CX3CL1 were unable to attract resting or activated T-cells from healthy donors; CCL5 could only attract resting T-cells, whereas CCL17 activated T-cells only. CXCL10 and CCL20 could attract both resting and activated T-cells from healthy donors. At this stage, patient recruitment resumed and the ability of CCL5, CXCL10 and CCL20 to attract psoriatic T-cells was evaluated. All three chemokines were able to induce significant migration by T-cells derived from patients with psoriasis; however, CCL20 was only able to do so at the highest concentration tested. Importantly, a larger percentage of psoriatic T-cells migrated towards CCL5, CXCL10, and CCL20, when compared to their healthy counterparts. These findings were in unison with our neutrophil data, reported earlier in this chapter, and suggest that T-cells, like neutrophils, might become primed for migration in the periphery. A study has shown that skin-homing T-cells in psoriasis express higher levels of CCR6 and show higher chemotactic responsiveness towards CCL20, when compared to healthy-donor derived cells[902]. Although this study has not addressed the factors affecting the increase in migration, it supports our findings. The observation that CCL5 can induce migration of patients with psoriasis-derived, but not healthy donor-derived activated, T-cells is important for the interpretation of the results. This finding demonstrates that the *ex vivo* activation of healthy T-cells cannot fully replicate the phenotype of T-cells derived from patients. Therefore, it is important to note that a definitive conclusion about the ability of CCL18, CCL27, and CX3CL1 to attract T-cells in the context of psoriasis cannot be drawn. Furthermore, in order to be able to make effective comparisons, further experiments need carried out. For example, the ability of CCL18, CCL27, and CX3CL1 to attract psoriatic T-cells should be assessed. Nevertheless, it has been previously demonstrated that skin-homing psoriatic T-cells express CCR6 at higher levels, when compared to receptors for other chemokines (e.g. CXCL10, CCL5)[902]. Considering these finding about CCL20 and its importance to the IL-23/Th17 axis in psoriasis, we next sought to determine T-cell migratory capacity in response to CCL20. Here we report that psoriatic T-cells have an enhanced responsiveness to CCL20 (200ng/mL), when compared to T-cells derived from healthy donors.

We also wanted to establish if this elevation in leukocyte migratory potential and chemokine responsiveness was unique to psoriasis. To do this we also isolated and migrated neutrophils and T-cells from patients with neutrophilic dermatoses and psoriatic arthritis. Our data showed that neutrophils, but not T-cells, derived from patients with NDs had an increase in their responsiveness to CXCL8 and CCL20, respectively. Pyoderma gangrenosum, a ND, is associated with abnormally high neutrophil numbers and elevated production of neutrophil-attracting molecules (e.g. CXCL8/1/2/3/16) in the skin lesions[903-905]. Furthermore, a T-cell imbalance, in favour of Th17 cells, has been described, but is unknown what chemokines may drive this[906]. Our data further highlights the similarities in pathogenesis that psoriasis and NDs share. This is important, as it might help inform the development of future therapies for the treatment of NDs. Nevertheless, it is important to note that we carried out our studies on a limited number of ND patients; however, these conditions are exceedingly rare, and even in an international neutrophilic-disease centre such as Leeds, suitable patients are challenging to recruit. Thus our data provide important early insights into ND pathogenesis. Furthermore, it would be interesting to examine the effects of Th17-derived cytokines on keratinocytes and production of neutrophil attracting factors in the context of NDs. In contrast, we report that neutrophils from patients with psoriatic arthritis exhibit an increase in their migratory potential and responsiveness to CXCL8. Our findings are in keeping with previous studies. For example, a study demonstrated that there is no difference between the in vivo function, including migration, of neutrophils from patients with psoriasis and psoriatic arthritis[907]. In contrast, psoriatic T-cells did not have an increased responsiveness to CCL20. This contradicts previous findings, which have shown an increased migration and accumulation of Th17 cells in the joints and synovial fluid of patients with PsA. It is important to note however, that most studies investigated the responsiveness of T-cells to patients' serum and synovial fluid. These substances will contain multiple chemokines and could therefore be more chemoattractive to T-cells, increasing the chances of detecting an increase in migration. We only investigated T-cell responsiveness to CCL20, which could, in the case of PsA, need to act in unison with other T-cell chemoattractants to induce migration. Nevertheless, it has been suggested that much like neutrophils, the T-cells from patients with PsA are more migratory than their healthy counterparts[908].

We next sought to start to explore the clinical context of these findings. The increase of migratory potential of psoriatic leukocytes was not related to the PASI score of patients, nor their age. Further to this migratory potential did not vary between males and females. However,

the recruitment of more patients may allow for those relationships to be better understood. It is likely that a larger a cohort of patients is needed for such an assessment. In addition to this, a larger pool of patients would allow us to characterise the low- and high-end responders better (e.g. Simpson's paradox). None of these questions have previously been addressed in the field. Finally, we examined the effect of different *in vivo* psoriasis treatment strategies on leukocyte migration. Treatment of patients with psoriasis with Apremilast, a PDE4 inhibitor, reduced the ability of their neutrophils to respond to CXCL8, but responsiveness of T-cells to CCL20 was unaffected. This is a novel mechanism of Apremilast on human cells, and of high clinical relevance given its licenced indictations. This is in keeping with previous reports of other (non-Apremilast), less specific, PDE4 inhibitors attenuating neutrophil migration, albeit not in vivo[664]. This prompted us to further investigate the effects of Apremilast on neutrophil migration in the next chapter. Interestingly, methotrexate was not able to reduce the responsiveness of neutrophils from patients with PsA to CXCL8. In contrast, methotrexate-induced inhibition of neutrophil migration has previously been noted in the context of rheumatoid arthritis[909]. Although our findings suggest that this might not be the case in PsA, it is important to note that we have investigated these effects on a very small cohort of patients. Nonetheless, our findings indicate there may be key differences between PsA and RA.

In conclusion, we have established an *ex vivo* transmigration system, utilising psoriatic leukocytes and psoriasis-associated chemokines, to study leukocyte migration in the context of psoriasis. With this *ex vivo* assay we have determined that not all chemokines are key to leukocyte plaque-homing and that psoriasis-derived leukocytes exhibit an enhanced migratory capacity. This assay will allow us to study leukocyte-stromal cell relationship and resulting migration in the context of psoriasis.

Chapter 4: The effect of Apremilast on neutrophil migratory potential in psoriasis

4.1 Contents

4.1 Contents

4.2 Introduction and hypotheses

In the previous chapter, we reported that the treatment of patients with psoriasis with Apremilast leads to the reduction of neutrophils' migratory capabilities. Apremilast is a drug which is used for the treatment of inflammatory conditions, such as rheumatoid arthritis and psoriasis[125,126]. It acts by inhibiting phosphodiesterase 4 (PDE 4) activity, which is an enzyme that breaks down cyclic adenosine monophosphate (cAMP)[126]. With remarkably little published evidence, it has been suggested that the increase in cAMP activity leads to the downregulation of pro-inflammatory mediators' expression, which in turn is responsible for alleviating disease symptoms[124-126]. However, there are no reports on how Apremilast affects leukocyte function and leukocyte migration in particular; it is also not clear what cellular and molecular changes by Apremilast treatment are responsible for limiting neutrophil migration defined in chapter 3. Most importantly, it would be of a great benefit to personalised medicine, if a link between drug treatment success and the attenuation of a specific biological function is elucidated. This would allow us to predict the success of a treatment strategy in advance and therefore limit the application of drug therapies which might not be as effective. Therefore, we decided to further investigate the effect of Apremilast on leukocyte migration *ex vivo* and define a possible molecular mechanism of action.

Apremilast's broad anti-inflammatory effects are largely attributed to its ability to downregulate the expression of multiple pro-inflammatory mediators (this is later explored in this chapter)[124-126]. However, Apremilast could also have a direct effect on neutrophils and their ability to migrate, by interfering with intracellular signalling pathways. It has been previously demonstrated that other less specific PDE4 inhibitors, can directly affect neutrophil function during lung inflammation and modulates expression of adhesion molecules and CXCL8-induced migration[664,910,911]. The effect of Apremilast on modulating neutrophil migration to CXCL8, and into inflamed skin, has not been examined before.

Therefore, **it was hypothesised that ex vivo treatment of neutrophils with Apremilast, reduces CXCL8 responsiveness. It was further hypothesised that Apremilast could potentially have an effect on the surface expression of CXCR2 - CXCL8 receptor – by neutrophils.** To test these hypotheses, we utilised our transwell migratory assay, (described in detail in the previous chapter) and flow-cytometry.

As discussed earlier, Apremilast has a broad anti-inflammatory effect, which includes the inhibition of IL-23, IL-17A and TNF expression[124-126]. These mediators are responsible for the

regulation and activation of neutrophil and stromal cell functions, which include chemokine receptor expression, cytokine and chemokine production. Further to this, it has been reported that Apremilast induces the heightened production of IL-10[912]. IL-10 is an anti-inflammatory cytokine, which has regulatory activities and has been shown to attenuate neutrophil recruitment to the site of inflammation[913]. This suggests that it is possible for Apremilast to subdue neutrophil migratory potential, and therefore plaque infiltration, indirectly. This could be achieved by limiting chemokine production and neutrophil exposure to pro-inflammatory mediators. Furthermore, it would suggest that Apremilast-related reduction of psoriasiform inflammation could, in part, be attributed to subdued neutrophil responsiveness. Based on this, **it was hypothesised that Apremilast restricts neutrophil influx into psoriasiform skin; reduces expression of key pro-inflammatory mediators and psoriasiform inflammation**.

In order to investigate the effect of systemic Apremilast administration on neutrophil influx into plaques and psoriasiform inflammation, we utilised the imiquimod mouse model of psoriasis. Imiquimod is a Toll-like receptor 7 (TLR7) (and TLR8 in humans) agonist, which is typically used for the treatment of cutaneous warts in humans[841]. However, the repeated topical application of imiquimod has become a widely used method for the induction of psoriasiform inflammation in mice[721]. The imiquimod mouse model has become a preferred research tool as it mirrors many of the pathways involved in psoriasis pathogenesis. For example, histopathological changes (e.g. compromised epidermal integrity, keratinocyte apoptosis, erythema, scaling, and skin thickening), cytokine expression profile (e.g. IL-17/23 axis), and cellular infiltrate[210,721]. Importantly, the imiquimod mouse model allows for the induction of psoriasiform inflammation at discrete skin sites, such as back skin and legs. Furthermore, this murine model of psoriasis involves the recruitment of neutrophils and formation of Munro's microabscesses at inflamed sites[847]. Therefore, enabling the detailed analysis of Apremilast treatment on neutrophil function in a psoriasis *in vivo* setting.

Importantly, Apremilast is not always effective at treating psoriasis, as some patients are not responsive to treatment[175]. Therefore, it is important to better understand the intracellular signalling pathways, which are induced by the drug and result in the reduction of inflammation. It has been suggested that PDE4 inhibitors supress neutrophil migration via the cAMP-dependent activation of downstream enzymes, such as protein kinase A (PKA)[160,161]. For example, PKA can induce the inhibition of cell surface expression of integrins and subsequent leukocyte migration[654,664,687]. However, due to the duality of the cAMP system and its effector proteins, the identification of an exact neutrophil migration inhibiting mechanism has been challenging. It

has also been demonstrated that cAMP-elevating agents inhibit intracellular Ca^{2+} fluxes in leukocytes[685,687-689]. This is an important finding as calcium intracellular levels become elevated upon chemokine receptor ligation[682,685,686]. Multiple studies have shown that intracellular calcium has a critical role in directional and transendothelial leukocyte and neutrophil migration[682,685,686]. For example, the removal of calcium from neutrophils inhibits neutrophil migration. Furthermore, elegant light-sheet microscopy studies in zebra fish have shown that calcium influx into the leading edge of the cell guides migration[682]. More recently, it has been shown that Rolipram, a PDE4 inhibitor, enhances cAMP-dependent re-sequestration of cytosolic Ca^{2+} in response to chemotactic peptide[687]. The data suggested that this is a result of up-regulated cyclic AMP-dependent PKA-activatable endomembrane Ca^{2+}-ATPase activity. However, no direct link between the cAMP-signalling pathway, Ca^{2+} fluxes and leukocyte migration has been previously identified.

These findings prompted us to **hypothesise that the *ex vivo* treatment of neutrophils with Apremilast abolishes cells' intracellular calcium flux in response to CXCL8.** To study calcium transients, we utilised *in vitro* staining of neutrophils with the intracellular Ca^{2+} fluorescence indicator Fluo4.

To date, no one has investigated the relationship between the effects of Apremilast treatment on neutrophil migration, neutrophil skin infiltration and psoriasis prognosis. Importantly, there is the possibility that Apremilast-induced reduction of psoriasiform inflammation could, in part, be due to subdued neutrophil influx and responsiveness. Uncovering a direct effect of Apremilast on leukocytes, important to the inflammatory process in psoriasis, could be a useful tool for predicting a patient's responsiveness to the drug. Determining if a drug would be efficacious in treating a patient could be critical for achieving optimal treatment results and could save money.

4.3 Chapter aims

- To determine the effect of Apremilast on neutrophil responsiveness to a key psoriasis-related chemokine - CXCL8
- To investigate Apremilast's mode of action in restricting neutrophil migration
- To investigate the effect of Apremilast on leukocyte migration in the Imiquimod murine model of psoriasis

4.4 Results

4.4.1 The *ex vivo* effect of Apremilast treatment on neutrophil migration

Treatment with Apremilast alleviates psoriasis symptoms by inhibiting PDE4. It has been suggested that this via a reduced expression of pro-inflammatory mediators, such as IL-17, IL-23, TNF and others[124-126]. It has been reported that PDE4 inhibition affects leukocyte migration, neutrophil migration in particular, and that PDE4 inhibitors might exert these effects directly[682,685,686]. These studies, however, did not involve psoriasis and utilised other, less specific than Apremilast, PDE4 inhibitors. Here, we exclusively focus on identifying the effects of Apremilast on psoriatic neutrophil responsiveness to CXCL8.

For the purpose of testing our hypotheses, the transwell transmigration assay, described in chapter 3, was utilised. To investigate the *in vivo* effect of Apremilast on neutrophil and T-cell migration, leukocytes were derived from Apremilast-treated patients with psoriasis and leukocyte migratory responsiveness towards CXCL8 or CCL20 was defined. To help inform the reader why this chapter primarily focuses on neutrophil migration, here we reiterate part of the findings that were presented in Chapter 3.4.4. These findings being that the treatment of patients with psoriasis with Apremilast, reduced the responsiveness of patients' neutrophils to key psoriasis-associated chemokine – CXCL8 (Figure 3.11 A). On the contrary, the responsiveness of T-cells, derived from Apremilast-treated patients with psoriasis, to another key psoriasis-associated chemokine – CCL20, did not subside significantly (Figure 3.11 B). Therefore, the effect of Apremilast on T-cell migration was not further investigated and will not be discussed in detail in this chapter.

Based on the finding that patients with psoriasis, treated with Apremilast, have reduced neutrophil responsiveness to CXCL8, we investigated the *ex vivo* effect of Apremilast on neutrophil migration. For this purpose, neutrophils were derived from untreated patients with psoriasis (i.e. psoriatic neutrophils). These neutrophils were treated with rising concentrations (as per previous PDE4 inhibitor studies and likely concertation of Apremilast in human plasma in treated individuals[175a,175b]) of Apremilast (0, 0.1, 1, 10, 100μM), for two hours, and ability to migrate to CXCL8 defined. Here, we report that the *ex vivo* treatment of neutrophils, derived

from healthy donors, with Apremilast did not result in reduced CXCL8 responsiveness (Figure 4.1 A). On the contrary, the ex vivo treatment of psoriatic neutrophils with 100μM Apremilast resulted in significant reduction of responsiveness to CXCL8 (Figure 4.1 B). It should be noted that pre-treatment with Apremilast concentrations lower than 100μM did not result in significant reduction of neutrophil responsiveness to CXCL8. It should be taken into account that cell viability was assessed by trypan blue stain only. The toxicity of Apremilast at as high doses as 100 μM could impact neutrophil viability and ability to migrate. Nevertheless, the data presented here suggest that Apremilast has a direct effect on neutrophil migration and responsiveness to CXCL8.

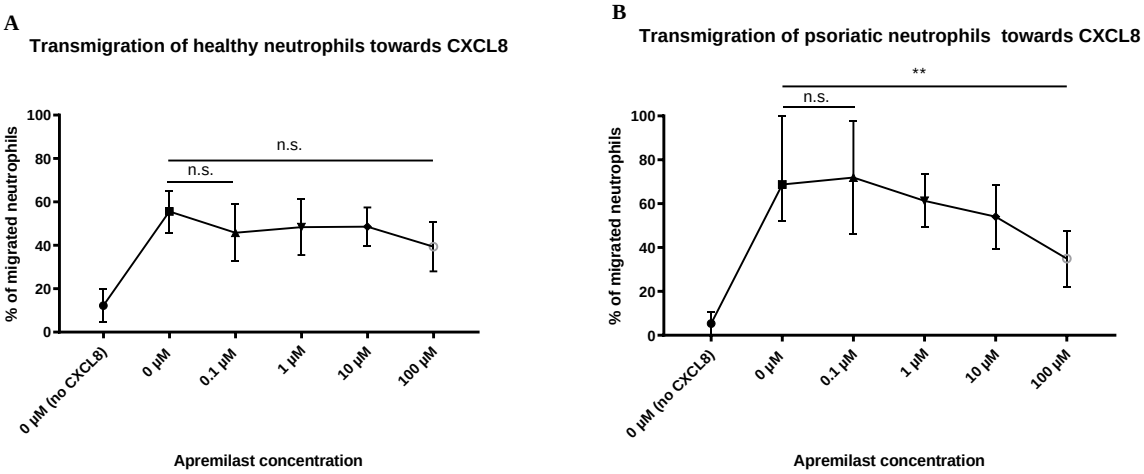


Figure 4.1| The *ex vivo* treatment of psoriatic, but not healthy, neutrophils with Apremilast, significantly reduced the responsiveness of neutrophils towards CXCL8

*Transwell analysis of the migratory capacity of Apremilast ex vivo treated, (A) healthy and (B) psoriatic neutrophils induced by 50ng/ml CXCL8. Following a 2h incubation at 37°C with neutrophil media or neutrophil media + 100µM Apremilast, cells were placed on transwell chambers (6.5mm, 3µM pores), migrated cells were collected and counted. Data are presented as interconnected dot plots, with each dot representing the mean of 6-7 biological replicates, with the SD (n= 6 healthy and 7 psoriasis) (One-way ANOVA test; **p<0.01; n.s. = non-significant).*

4.4.2 Apremilast restricts PMN influx into psoriasiform skin in the imiquimod mouse model of psoriasis

As discussed earlier, Apremilast can inhibit the production of multiple pro-inflammatory molecules and thus affect neutrophil function indirectly[124-126]. We have, thus far, demonstrated that systemic *in vivo* treatment with Apremilast can subdue neutrophil responsiveness to CXCL8 in humans with psoriasis. Therefore, we sought to investigate the effect of systemic Apremilast treatment on neutrophils in a greater detail. To further evaluate the effect of systemic Apremilast treatment *in vivo*, we wanted to investigate whether Apremilast treatment specifically reduces neutrophil influx into inflamed psoriasiform skin and whether this affects 'clinical' outcome.

In order to study this in a psoriasis *in vivo* setting, the imiquimod mouse model of psoriasis was utilised. As discussed earlier, imiquimod-induced skin inflammation is the most widely used and validated mouse model for studying psoriasis[210,721]. This is because it bears significant histological and molecular similarities to human psoriasis and can be induced at pre-determined skin sites. It is particularly useful for this study, as it involves the recruitment of neutrophils to the IMQ-induced plaques.

In this study, the detection of neutrophils from mouse skin was carried out via flow-cytometry. Prior to this, however, an antibody staining panel had been selected and optimised. The following markers were selected: CD45 (present in all cells of the hematopoietic lineage except erythrocytes and plasma cells), CD11b (myeloid lineage cell marker), Ly6C and Ly6G (cells that have a high Ly6G and an intermediate Ly6C expression are regarded as neutrophils), DRAQ7 (a dead cell stain). Two different combinations of fluorophore-conjugated antibodies, against the targets outlined above, were selected and tested. Antibody combination 1 consisted of the following antibodies: 1) anti-Ly6G (BV421); 2) anti-Ly6C (PE); 3) anti-CD45 (PE-Cy5); 4) anti-CD11b (PE-Cy7). Antibody combination 2 consisted of: 1) anti-Ly6G (APC); 2) anti-Ly6C (PE); 3) anti-CD45 (PB); 4) anti-CD11b (PE-Cy7). For the purpose of this optimisation experiment, cells to be stained were extracted from mouse femur bone marrow. Flow-cytometric analysis showed that both panels were able to stain neutrophils and therefore allow us to identify the cells (Figure 4.2). Antibody combination 1 was chosen and used throughout this study.

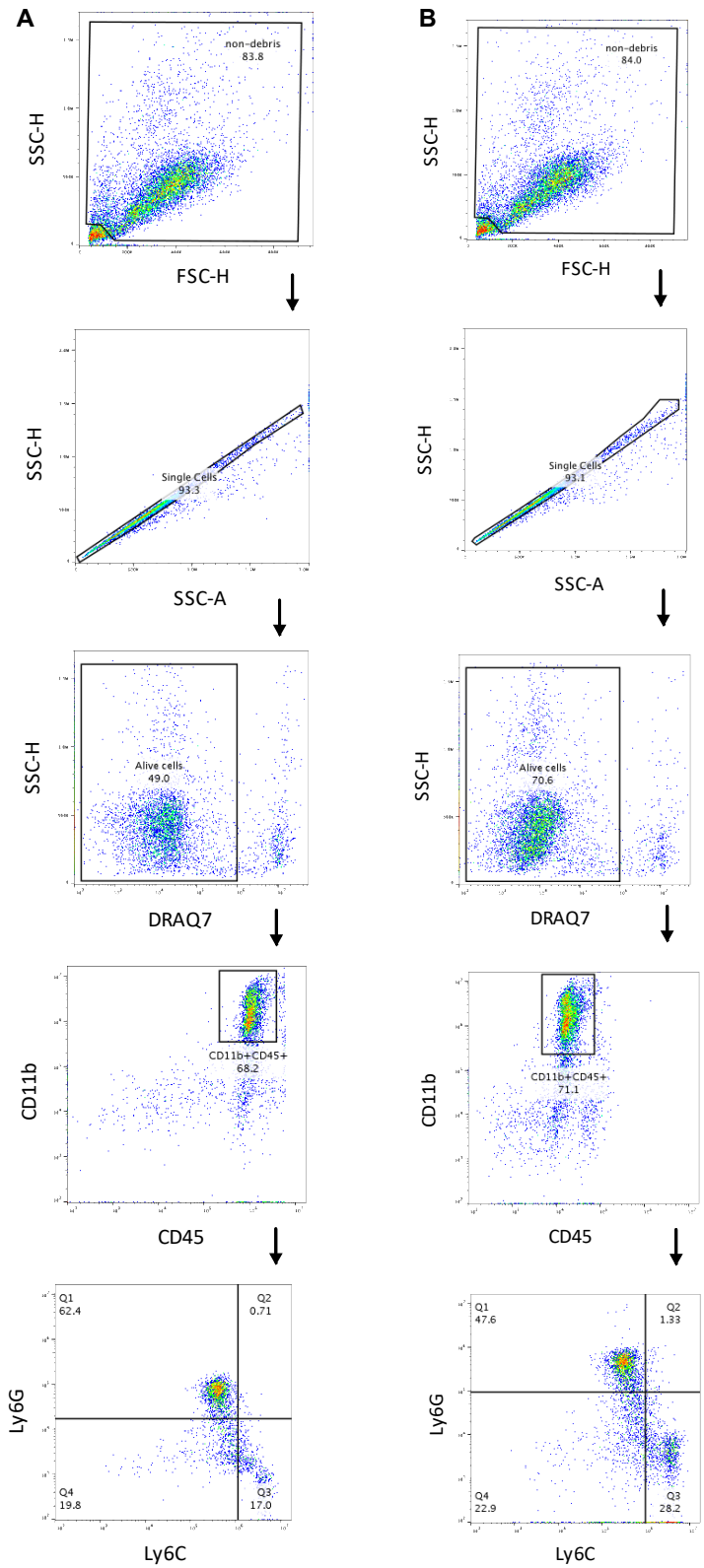

A
B
non-debris
83.8
non-debris
84.0
SSC-H
FSC-H
Single Cells
93.3
Single Cells
93.1
SSC-A
Alive cells
49.0
Alive cells
70.6
DRAQ7
CD11b+CD45+
68.2
CD11b+CD45+
71.1
CD11b
CD45
Q1
62.4
Q2
0.71
Q4
19.8
Q3
17.0
Q1
47.6
Q2
1.33
Q4
22.9
Q3
28.2
Ly6G
Ly6C

Figure 4.2| Optimisation of antibody staining panel for the identification of neutrophils derived from mice

Flow-cytometric analysis showing the identification of neutrophils from mouse bone marrow cells, achieved by staining with two different antibody combinations (AbC). AbC 1 (A) contains the following fluorophore-conjugated antibodies: 1. anti-Ly6G-BV421; 2. anti-Ly6C-PE; 3. anti-CD45-PECY5; 4. anti-CD11b-PECy7 (B) contains the following fluorophore-conjugated antibodies: 1. anti-CD45-PB; 2. anti-Ly6C-PE; 3. anti-CD11b-PECy7; 4. anti-Ly6G-APC; Dead cell stain – DRAQ7 – was added to both combinations. Cells that have a high Ly6G and an intermediate Ly6C expression are regarded as neutrophils.

The original work that described the imiquimod mouse model, induced psoriasiform inflammation in the back skin of mice[721]. However, work in the McKimmie group has shown that it is difficult to obtain large numbers of viable cells from inflamed back skin. In comparison, foot skin, which is thinner and can be more easily digested, resulting in substantially less cell death. Therefore, here we wanted to determine which skin site, when inflamed with topical imiquimod, could be best digested to enable the identification of viable neutrophils post skin digestion. For the purpose of this experiment, we induced inflammation in both shaved back skin and skin from the upper side of the foot. This was done by the single application of 5% imiquimod cream (62.5mg), which leads to neutrophil influx in 24h. In this optimisation experiment, we were not aiming to induce psoriasiform inflammation, which requires multiple cream applications. Instead, we wanted to achieve a quick imiquimod-driven neutrophil influx, minimising animal suffering. As this is a technical optimisation, the experiment was carried out using one imiquimod-treated mouse and two control mice. The gating strategy applied in this flow-cytometric analysis is displayed in Figure 4.3. Here, we show that the single cell suspension derived from mouse foot skin contained viable $Ly6G^{hi}Ly6C^{int}$ cells. Whereas, this was not true for mouse back skin-derived cell suspension (Figure 4.4), likely as a result of inefficient skin digestion. Therefore, foot skin was also a pre-determined skin site for the induction of psoriasiform inflammation in this particular study.

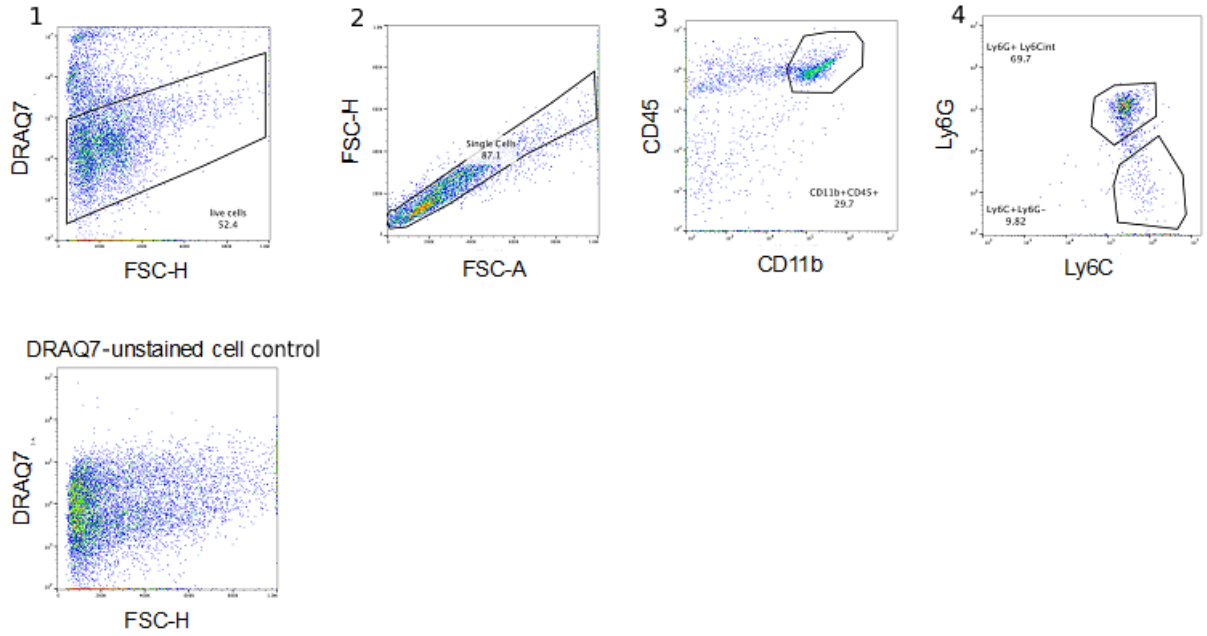


Figure 4.3| Flow-cytometric gating strategy for the identification of viable Ly6G^{hi}Ly6C^{int} cells from digested mouse skin.

This gating strategy was carried out on technical positive control, which was mouse skin cells enriched with mouse bone marrow cells (positive control). Cells were derived from mouse skin lysates and stained for CD45, CD11b, Ly6G, Ly6C, and DRAQ7. In (1) Events were gated on Forward side scatter and DRAQ7 to exclude debris and dead cells; in (2) doublet cells were excluded by gating on forward side scatter height vs area; in (3) leukocytes were identified on the basis of CD45 and CD11b expression; in (4) neutrophils were identified on the basis of Ly6G^{high} and Ly6C^{int} expression. The bottom panel represents the unstained DRAQ7 control, which helped define the DRAQ7 gate.

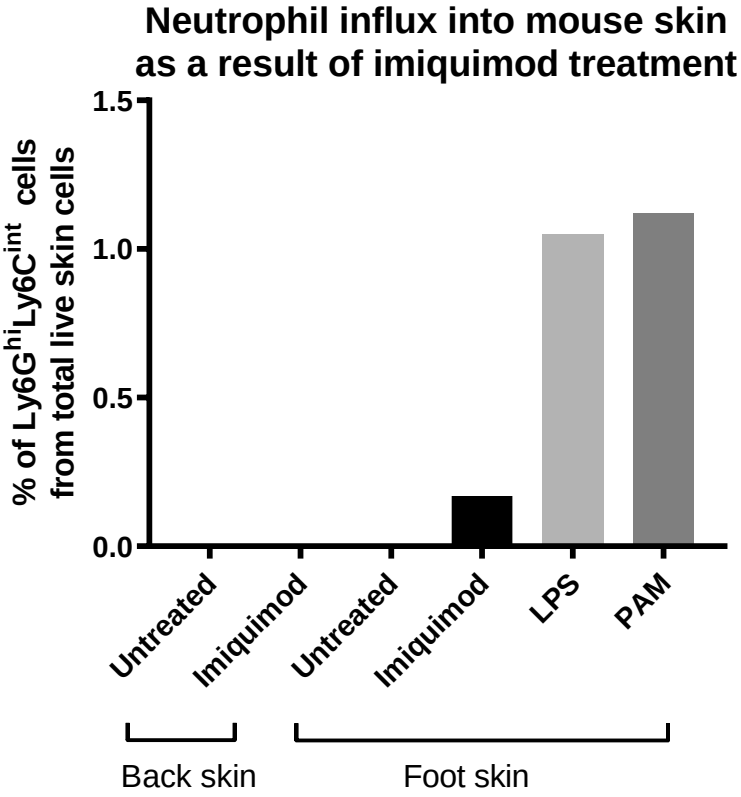


Figure 4.4| Optimisation of a pre-determined skin site for the induction of psoriasiform inflammation by topical application of imiquimod cream in mice

Flow-cytometric analysis of Ly6G^{hi}Ly6C^{int} cells from imiquimod-treated and untreated back or foot skin in mice. Following skin digestion and single cell suspension acquisition, cells were stained with antibodies against Ly6G, Ly6C, CD45, CD11b. Dead cell stain marker - DRAQ7 – was added. Foot skin treated with Lipopolysaccharide (LPS) or Pam3CSK4 (PAM) (topical administration) was used as a positive control. Untreated skin was used as a negative control. n=1(imiquimod-treated mice), n=2(controls). Data are presented as bar graphs.

Next, to define whether Apremilast treatment modulates the number of neutrophils infiltrating psoriasiform inflamed skin, the imiquimod model of psoriasiform inflammation was induced. This was done in C57BL/6 mice by the repeated application of 5% imiquimod cream (62.5mg) (Aldara™, IMQ) to pre-determined skin sites for up to 4 days. This methodology was in keeping with the original work that described this model[721]; however, in addition to inducing inflammation to the central shaved back skin, foot skin was also inflamed, as per the paragraph above. Mice received an oral daily dose of 0.5mg Apremilast dissolved in carrier (0.5% carboxymethyl cellulose; 0.25% Tween 80), as per previous publications[917], or control carrier. The treatments were carried out as per the treatment schedule in Figure 4.5.

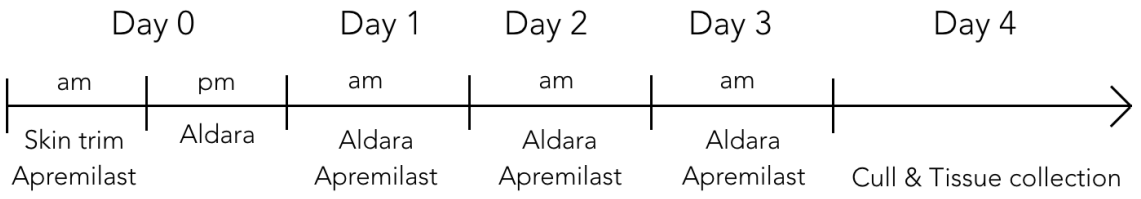


Figure 4.5| Experimental plan for the assessment of the effect of systemic Apremilast treatment on neutrophil influx into Imiquimod-induced psoriasiform skin.

On the day of the cull, tissues were collected and processed. Foot skin was digested and extracted cells were labelled with Antibody combination 1. Following the staining, samples were analysed via Flow-cytometry. The gating strategy applied in this flow-cytometric analysis is displayed in Figure 4.3. Here, we show that treatment of mice with Apremilast significantly reduced the influx of $Ly6G^{hi}Ly6C^{int}$ cells into imiquimod treated skin (Figure 4.6). Cells that have a high Ly6G and an intermediate Ly6C expression were regarded as neutrophils.

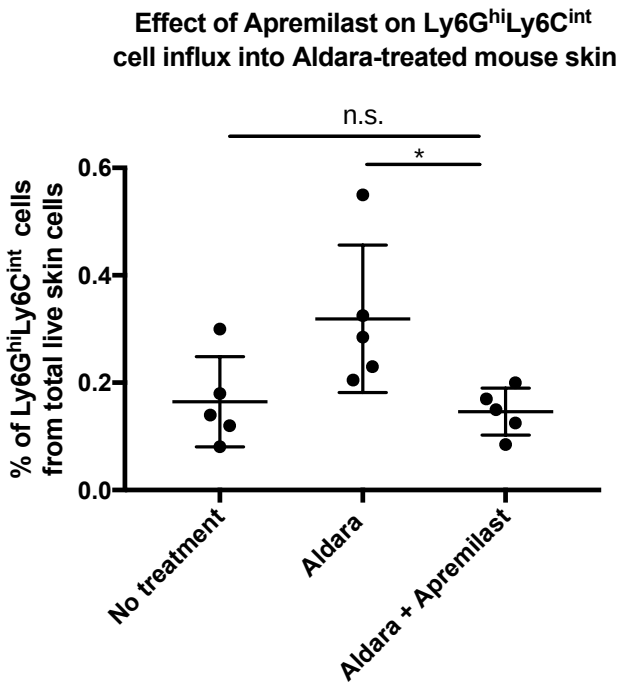


Figure 4.6| Oral administration of Apremilast to mice, over a 4-day period, reduced neutrophil influx into aldara-treated skin

*Flow cytometry-based analysis of the effect of Apremilast on Ly6G^{hi}Ly6C^{int} cell (neutrophil) influx into mouse skin. Three groups of mice were utilised. Group 'Aldara+Apremilast' received daily topical treatment with Aldara alongside a dose of 0.5mg Apremilast, per mouse, dissolved in carrier solution (0.5% carboxymethyl cellulose; 0.25% Tween 80) for up to 4 days. Group 'Aldara' received daily topical Aldara treatment and oral administration of carrier solution alone (no Apremilast) for up to 4 days; Group 'No treatment' received no Aldara, Apremilast or carrier. Skin was digested and cells were stained with fluorophore-conjugated antibodies against the following targets: CD11b, CD45, Ly6G, and Ly6C. Dead cell stain marker - DRAQ7 - was added. Cells were normalised against total live cells. Blind-folded analysis was applied. Data are presented as dot plots, with each dot representing the average of total live Ly6G^{hi}Ly6C^{int} cells from two feet of the same mouse, with the Mean+SD (n= 5 mice per group) (One-way *p<0.1; n.s. = non-significant).*

We next wanted to define what impact the reduction of neutrophil influx had on skin inflammation and whether this modulated clinical outcome. As mentioned earlier, psoriasiform inflammation was also induced on the shaved back skin of mice. Upon visual inspection of the back shaved portion of the skin, it was determined that Apremilast treatment resulted in a visibly less inflamed skin appearance (e.g. visibly less 'scaling' of the skin and reduced redness) (Figure 4.7). In order to quantify these skin changes, we utilised a comprehensive clinical scoring system, developed by Shams et.al, known as modified Psoriasis Area Severity Index (mPASI)[853]. This system is based on the human PASI scoring system and takes into account erythema, scaling and skin thickness. It is important to note that the mPASI score was recorded in alive non-anaesthetised animals, prior to cull. In keeping with skin appearance, the mPASI score in C57BL/6 mice treated with Apremilast was significantly lower than the mPASI score of Apremilast-untreated mice (Figure 4.8 A). Further to this, C57BL/6 mice treated with Apremilast experienced an insignificant weight loss (Day 1 vs Day 4). Whereas, Aldara-treated C57BL/6 mice that did not receive Apremilast lost a significant amount of weight over the course of 4 days (Figure 4.8 B). It is important to note that although Apremilast treatment aided the less dramatic increase in mPASI and weight loss, it did not result in a complete return to a healthy phenotype. This is evident by the significant increase of mPASI of Apremilast-treated mice, when compared to Aldara-untreated mice. Further to this, Aldara-untreated mice did not lose weight, whereas Apremilast-treated mice did, albeit insignificant.

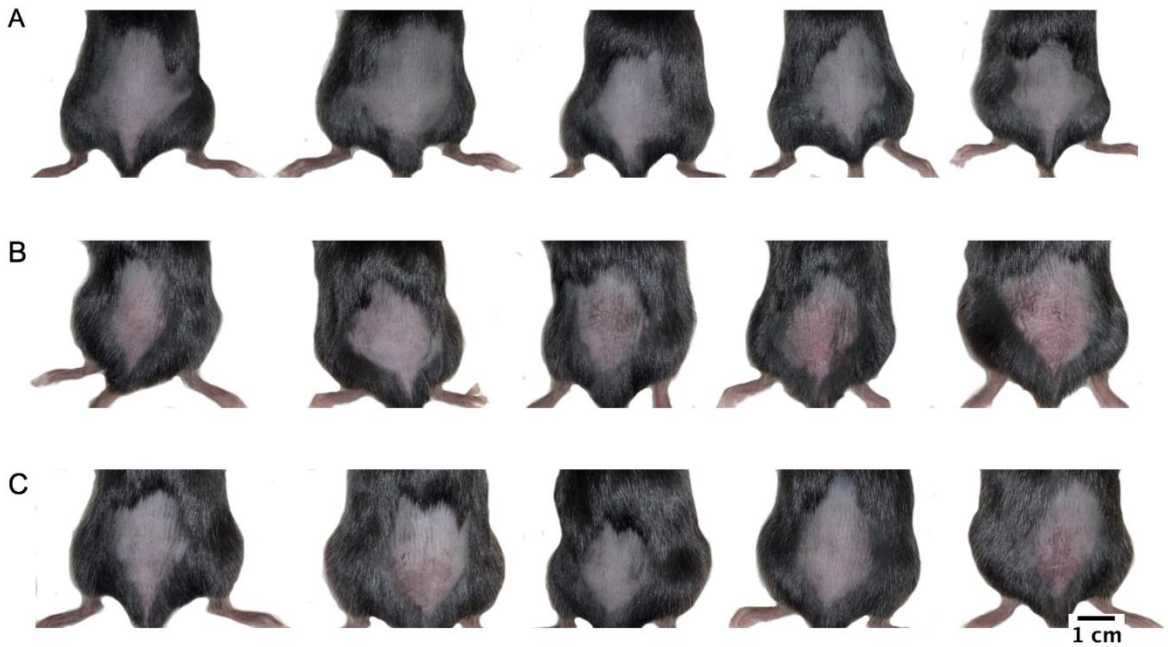


Figure 4.7| Apremilast visibly alleviated inflammation of Aldara-treated skin in C57BL/6 mice

Images showing the effect of Apremilast treatment on the inflammatory state of shaved back skin of three treatment groups of C57BL/6 mice. Scaling and redness of the skin are considered as visible signs of inflammation in this thesis. Psoriasiform inflammation was induced by the topical application of TLR7-agonist - Imiquimod (Aldara cream). (A) shows group 'No treatment', which received no Aldara, Apremilast or carrier. (B) shows group 'Aldara', which received daily topical Aldara treatment and oral administration of carrier solution alone (no Apremilast) for up to 4 days; (C) shows group 'Aldara+Apremilast', which received daily topical treatment with Aldara alongside a dose of 0.5mg Apremilast, per mouse, dissolved in carrier solution (0.5% carboxymethyl cellulose; 0.25% Tween 80) for up to 4 days. Images were taken shortly after cull. Scale bar (1cm) was added using Fiji ImageJ.

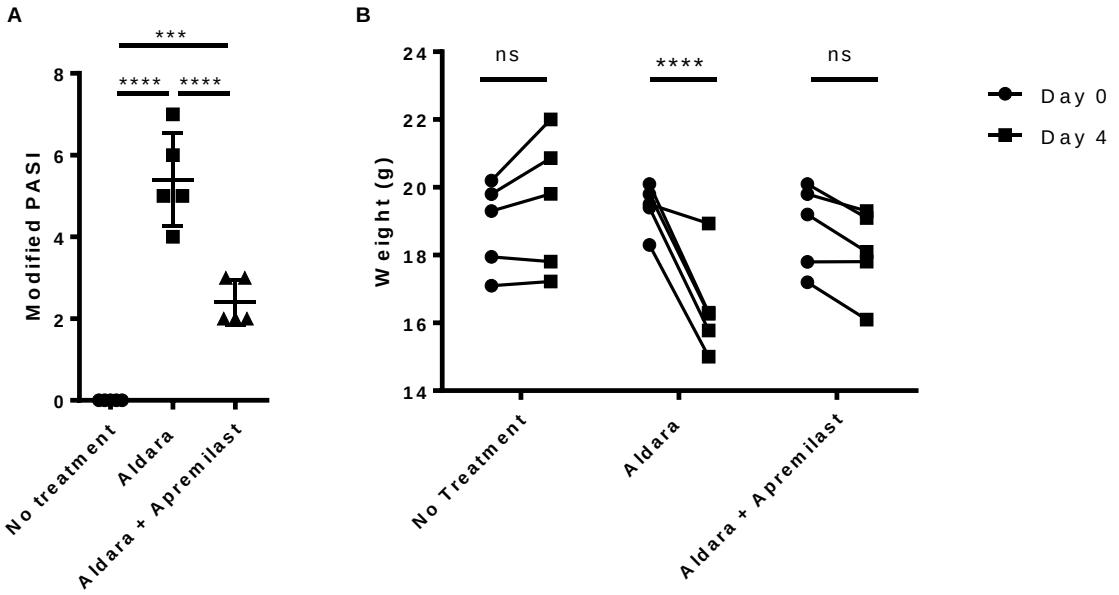


Figure 4.8| C57BL/6 mice, with Aldara-induced psoriasiform inflammation, treated with Apremilast had a reduced mPASI score and were saved from a significant weight loss, when compared to Apremilast-untreated mice

*Graphs showing the effect of Apremilast treatment on modified PASI score (A) and weight (B) of mice with psoriasiform inflammation. Psoriasiform inflammation was induced by the topical application of TLR7-agonist - Imiquimod (Aldara cream). Group 'Aldara+Apremilast' received daily topical treatment with Aldara alongside a dose of 0.5mg Apremilast, per mouse, dissolved in carrier solution (0.5% carboxymethyl cellulose; 0.25% Tween 80) for up to 4 days. Group 'Aldara' received daily topical Aldara treatment and oral administration of carrier solution alone (no Apremilast) for up to 4 days; Group 'No treatment' received no Apremilast, Apremilast or carrier. Modified PASI was assessed on the day of culling, whereas weight was recorded daily. Data collection and analysis carried out blind-folded. Data in (A) are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (5 mice per group) (One-way ANOVA test; ***p<0.001; ****p<0.0001). Data in (B) are presented as dot plots, with each dot representing a different biological sample on day 0 and day 4 (connected dots signify the same animal) (5 mice per group) (Two-way ANOVA test; ****p<0.0001; n.s. = non-significant.).*

Following the discovery that treatment of mice with Apremilast can reduce the degree of neutrophilic influx and clinical outcome to Aldara application, the next step was to determine whether inflammatory gene expression and histopathological markers of psoriasiform inflammation, induced by Aldara, were also changed by Aprelimast treatment. To do this, mouse back skin was RNAlater-treated, and RNA was extracted from it for cDNA synthesis and a subsequent qPCR gene expression analysis. We found that the expression of pro-inflammatory genes, such as TNF, IL-1, IL-6, CCL2, CXCL1, and CXCL2 did not change significantly after induction of psoriasiform inflammation or treatment with Apremilast (Figure 4.9). However, it is important to note that this is an acute model of the disease. Therefore, cytokine production is transient and previous work has also reported difficulty to detect cytokine expression[210,721,754]. Further to this, the qPCR technique used in this experimental setting could have failed to work (on the basis of TATA-binding protein gene expression levels). In addition to studying the gene expression profile of psoriasiform skin, formalin-treated skin was taken for processing, embedment, sectioning and H&E staining. A representative image for each H&E-stained sample can be seen in Figure 4.10. Upon visual inspection, it was determined that the epidermis of Apremilast-treated mice was visibly smaller in thickness. However, in order to more accurately determine changes in inflammatory state and quantify results, the epidermal thickness was measured, and neutrophils were also enumerated. The strategy for doing this can be found in Figure 4.11. Here, we demonstrate that, in conjugation with our earlier findings defined by flow cytometry, Apremilast treatment of mice with psoriasiform inflammation reduced neutrophil influx into skin (Figure 4.12 A). Further to this, the epidermal thickness of Apremilast-treated mice was significantly reduced, when compared to Apremilast-untreated mice with psoriasiform inflammation (Figure 4.12 B). These data, together with the flow cytometric analysis of skin cells, suggest for an Apremilast-induced reduction of neutrophilic influx and inflammatory state of skin with psoriasiform inflammation.

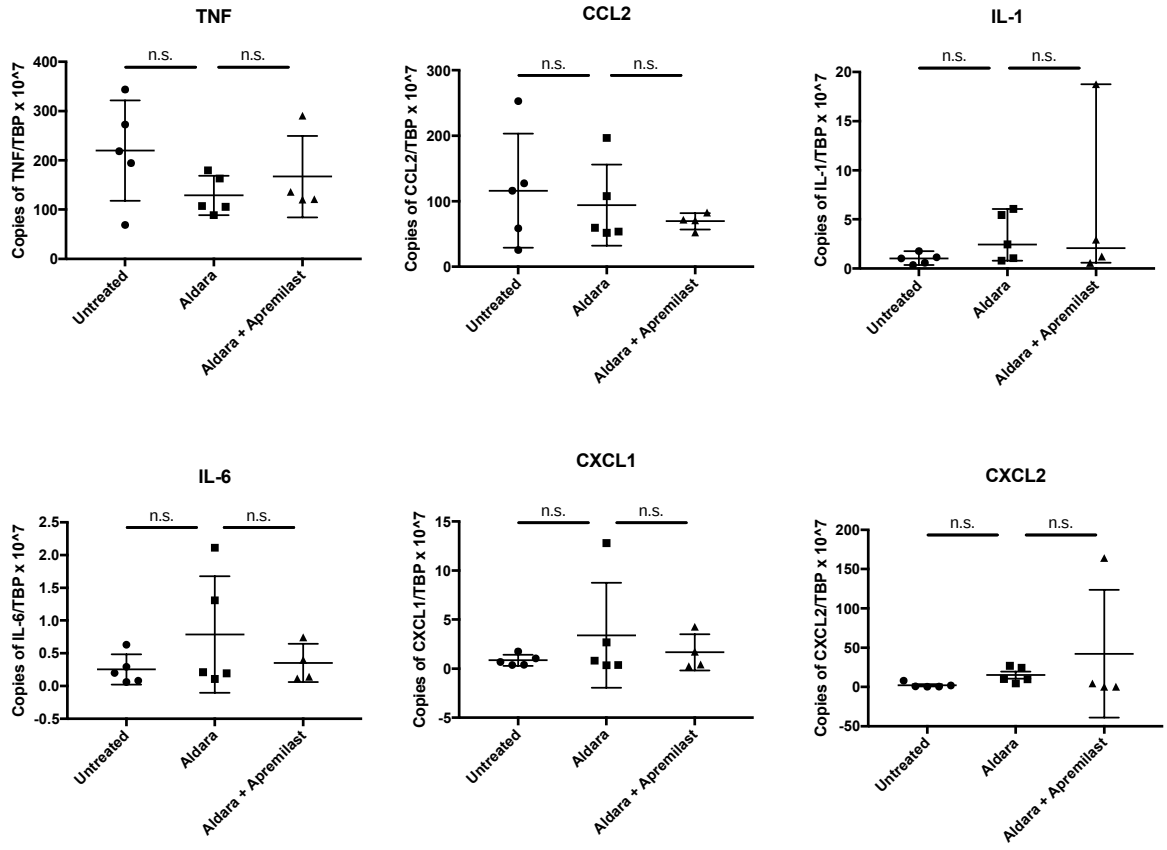


Figure 4.9| The expression of TNF, CCL2, IL-1, IL-6, CXCL1, and CXCL2 in the skin of C57BL/6 mice was not affected by topical Aldara application or/and treatment with Apremilast

Graphs showing real-time qPCR analysis of the effect of Apremilast treatment on the expression of TNF, CCL2, IL-1, IL-6, CXCL1, and CXCL2, in the skin of C57BL/6 mice with psoriasiform inflammation. Psoriasiform inflammation was induced by the topical application of TLR7-agonist - Imiquimod (Aldara cream). Group 'Aldara+Apremilast' received daily topical treatment with Aldara alongside a dose of 0.5mg Apremilast, per mouse, dissolved in carrier solution (0.5% carboxymethyl cellulose; 0.25% Tween 80) for up to 4 days. Group 'Aldara' received daily topical Aldara treatment and oral administration of carrier solution alone (no Apremilast) for up to 4 days; Group 'No treatment' received no Apremilast, Apremilast or carrier. Data in are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (4-5 mice per group) (One-way ANOVA test; n.s. = non-significant.).

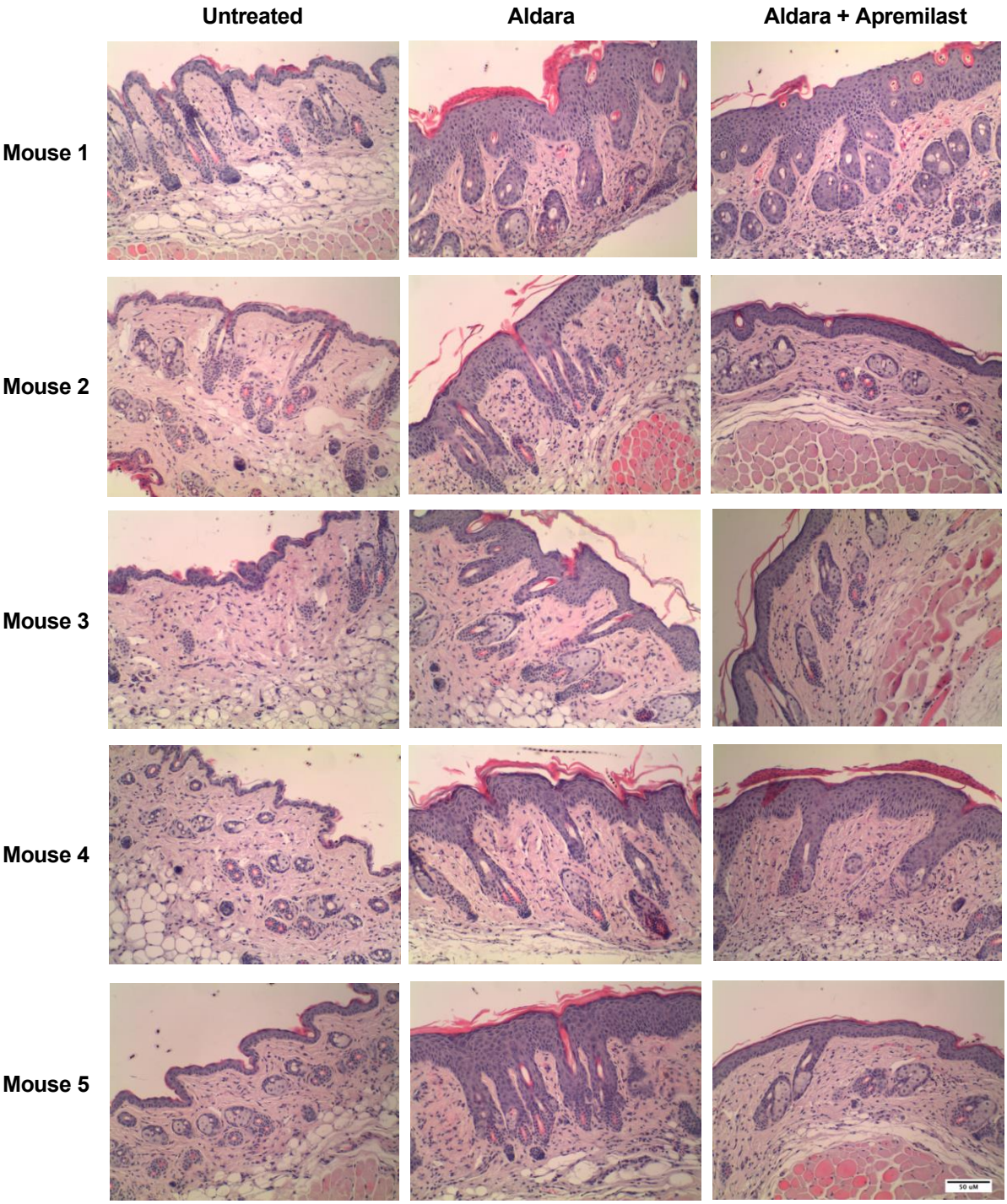

Untreated
Aldara
Aldara + Apremilast
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
50 uM

Figure 4.10| Treatment with Apremilast visibly reduced psoriasiform inflammation in the skin of C57BL/6 mice

Images of H&E stained C57BL/6 mouse skin samples showing the effect of Apremilast treatment on the inflammatory state of Aldara-treated mouse skin. Psoriasiform inflammation was induced by the topical application of TLR7-agonist - Imiquimod (Aldara cream). Group 'Aldara+Apremilast' received daily topical treatment with Aldara alongside a dose of 0.5mg Apremilast, per mouse, dissolved in carrier solution (0.5% carboxymethyl cellulose; 0.25% Tween 80) for up to 4 days. Group 'Aldara' received daily topical Aldara treatment and oral administration of carrier solution alone (no Apremilast) for up to 4 days; Group 'No treatment' received no Apremilast, Apremilast or carrier. 5 mice per group. Each image is a representative of a set of three images, each captured at a random skin sample site. Scale bar (50μM) was added using Fiji ImageJ.

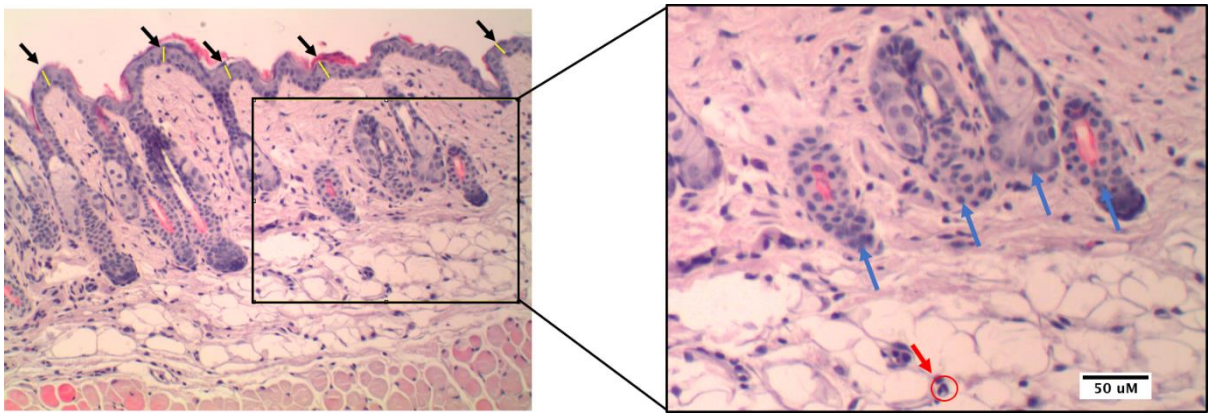


Figure 4.11| Strategy for taking epidermal thickness measurements and counting of neutrophils in H&E stained samples of mouse skin

H&E staining in the skin of a C57BL/6 mouse, which has not been treated with Aldara or Apremilast. Images are showing the strategy used for the analysis of epidermal thickness and neutrophilic influx into the skin of mice. On the left panel is an image, a representative of a set of three images, taken at a magnification of 20X, at a random tissue slide location. The yellow lines with black arrows pointed at them, represent the epidermal thickness measurements taken – 5 random measurements per image (3 images per mouse, 15 measurements per mouse). Neutrophil counting was carried out on a total area of 150μM X 200μM from the original image and is displayed on the right panel. Cells that resembled the cell within the red circle on the right panel were considered neutrophils. Cells within hair follicle structures (indicated by the blue arrows on the right panel) were not counted. Scale bar: 50μM.

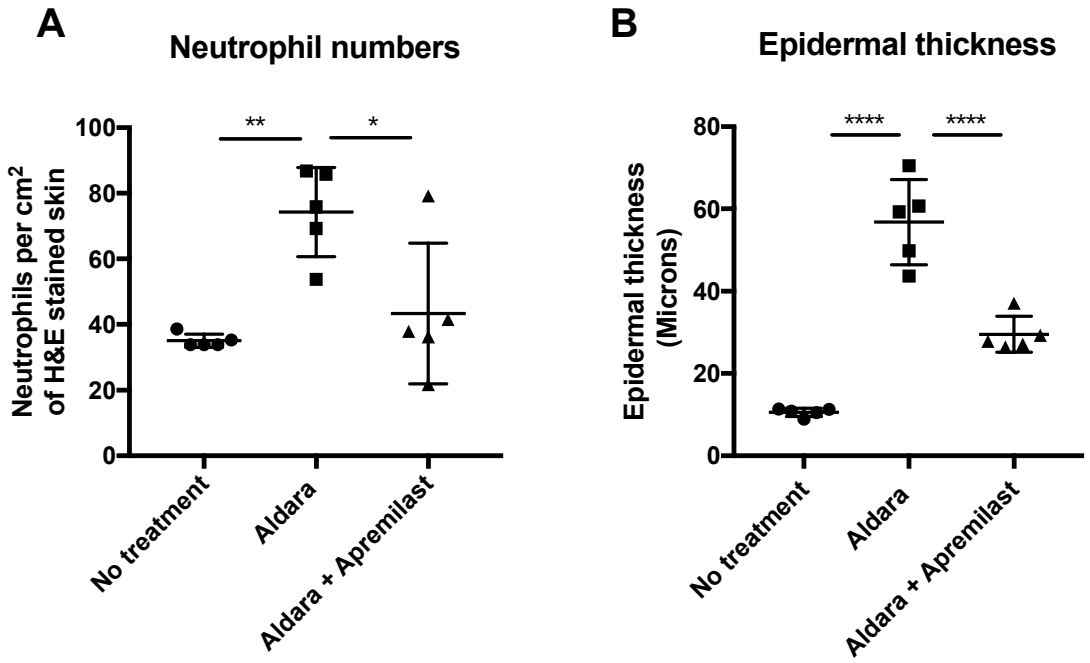


Figure 4.12| C57BL/6 mice, with Aldara-induced psoriasiform inflammation, treated with Apremilast had a reduction in epidermal thickness and neutrophil skin numbers, when compared to Apremilast-untreated mice

*Graphs showing the effect of Apremilast treatment on neutrophil skin numbers (A) and epidermal thickness (B) of mice with psoriasiform inflammation. Psoriasiform inflammation was induced by the topical application of TLR7-agonist - Imiquimod (Aldara cream). Group 'Aldara+Apremilast' received daily topical treatment with Aldara alongside a dose of 0.5mg Apremilast, per mouse, dissolved in carrier solution (0.5% carboxymethyl cellulose; 0.25% Tween 80) for up to 4 days. Group 'Aldara' received daily topical Aldara treatment and oral administration of carrier solution alone (no Apremilast) for up to 4 days; Group 'No treatment' received no Apremilast, Apremilast or carrier. The data represent the Mean+SD of values from 15 mice (5 per group). Each dot in (A) represents the average of 15 measurements (3 images per mouse, 5 measurements per image) per mouse; each dot in (B) represents the average of three counts (3 images per mouse, 1 count per image) per mouse. Data collection and analysis carried out blind-folded. Ordinary one-way ANOVA test; *p<0.5; **p<0.01; ****p<0.0001.*

4.4.3 Apremilast does not affects psoriatic neutrophil CXCR2 surface expression

As discussed earlier in this chapter, reports indicate that PDE4 inhibition leads to the subdued expression of important neutrophil surface molecules, such as the adhesion molecules (e.g. CD11b)[654,664,687]. This, in combination with the finding that Apremilast can inhibit neutrophil CXCL8 responsiveness directly, prompted an investigation on the effect of Apremilast on neutrophil CXCR2 – CXCL8 receptor – surface expression.

Neutrophils were derived from patients with psoriasis or healthy donors and were treated with 100μM Apremilast for two hours. This concentration of Apremilast was chosen as it reduced neutrophil responsiveness to CXCL8 significantly (Figure 4.1 B). Neutrophils were then stained with antibodies against CD15, CD16 and CXCR2. The flow cytometric analysis showed that Apremilast affects CXCR2 surface expression of healthy donor-derived (Figure 4.13 A), but not patient with psoriasis-derived (Figure 4.13 B), neutrophils. A trend towards apermilast-induced reduction in CXCR2 expression was noted in psoriatic neutrophils. The gating strategy for CXCR2+ neutrophils can be found in Figure 3.7.

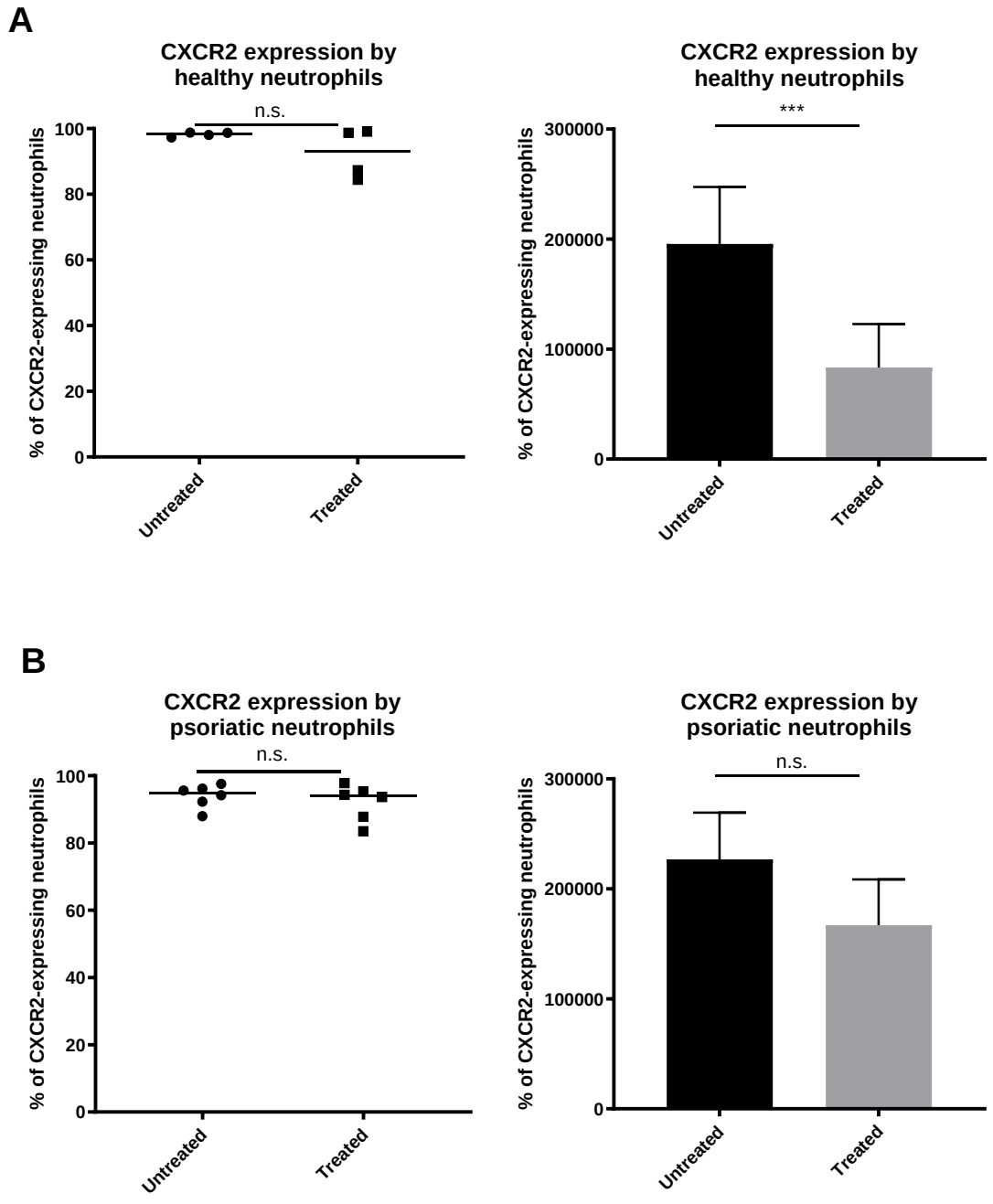


Figure 4.13| The *ex vivo* treatment of neutrophils with Apremilast, did not subdue surface expression of CXCR2 on psoriatic neutrophils

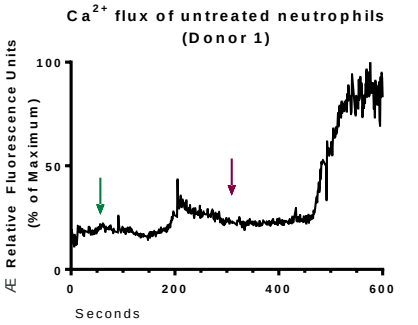
Flow-cytometric analysis of the CXCR2 surface expression of untreated or ex vivo Apremilast-treated neutrophils, derived from healthy donors (A) or patients with psoriasis (B). Following a 2h incubation at 37°C with neutrophil media (A) or neutrophil media + 100μM Apremilast (B), neutrophils were stained with antibodies against CD15, CD16 and CXCR2. Expression of CXCR2 is presented as CXCR2-expressing CD15+CD16+ cells / total CD15+CD16+ cells or mean fluorescence intensity (MFI). Data are presented as dot plots, with each dot representing a different biological sample or bar graphs, with the Mean+SD (4 healthy; 6 psoriasis samples) (paired t-test); n.s. = non significant

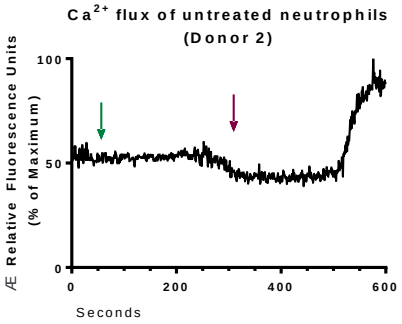
4.4.4 Apremilast can abolish CXCL8-induced intracellular calcium transients in neutrophils

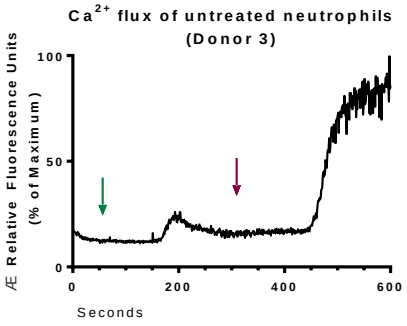
As Apremilast was discovered to affect neutrophil migration directly, we wanted to further investigate the mechanisms behind its action. Based on our findings and previous reports that PDE4 inhibition affects intracellular calcium flux and migration, we tested the effect of Apremilast on neutrophil calcium flux in response to $CXCL8^{685,687-689}$. Neutrophils were derived from patients with psoriasis or healthy donors and were treated with 100μM Apremilast for two hours. Following treatment, neutrophils were stained with the Ca^{2+} fluorescence indicator Fluo4 for 30 minutes and analysed straight after. Intracellular calcium transients in response to CXCL8 were measured via flow cytometry and were compared to a positive control - calcium transients generated by Ionomycin. Ionomycin is a calcium ionophore, which enhances Ca^{2+} influx by stimulating store-regulated cation entry.

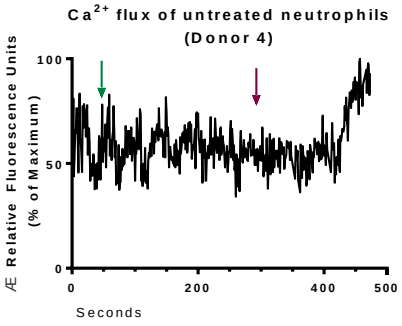
The flow-cytometric analysis showed that CXCL8-induced intracellular Ca^{2+} fluxes of psoriatic neutrophils are more pronounced than those in healthy cells (Figure 4.14 and 4.15). Furthermore, this is in agreement with the findings, described in chapter 3, that psoriatic neutrophils are more responsive towards CXCL8 than healthy neutrophils. The flow-cytometric analysis demonstrated that pre-treatment of neutrophils with Apremilast can abolish neutrophil intracellular Ca^{2+} flux in response to CXCL8. This was true for both healthy and psoriatic neutrophils (Figure 4.14 and 4.15); however, this was clearer in psoriatic neutrophils, due to their more CXCL8-responsive nature. Interestingly, untreated neutrophils derived from healthy donor 4 (Figure 4.14) and patient 3 (Figure 4.15) did not exhibit Ca^{2+} transients when stimulated with CXCL8. This was also true for their Apremilast-treated counterparts. Further to this, the CXCL8-induced Ca^{2+} flux of neutrophils derived from patients 1&5 (Figure 4.15) was not affected by Apremilast treatment. Interestingly, the Ca^{2+} flux of Apremilast-treated neutrophils in response to ionomycin, also appeared to be delayed in some of the cases, when compared to the untreated counterparts. This was true for both healthy (Figure 4.14) and psoriatic neutrophils (Figure 4.15). The Ionomycin-induced Ca^{2+} transients of Apremilast-treated psoriatic neutrophils was diminished in one of the cases (Figure 4.15). However, this phenomenon is independent of Apremilast mode of action, as ionomycin is an ionophore. Importantly, the flow cytometric calcium flux data is qualitative, as 'hard' to define quantitatively.

A

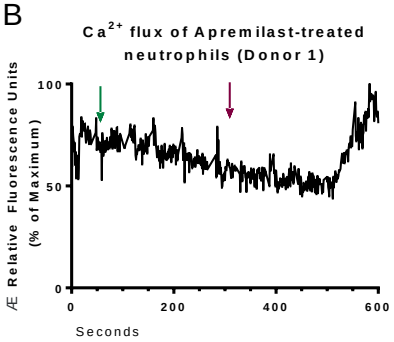

Ca2+ flux of untreated neutrophils
(Donor 1)
Æ Relative Fluorescence Units
(% of Maximum)
100
50
0
0
200
400
600
Seconds

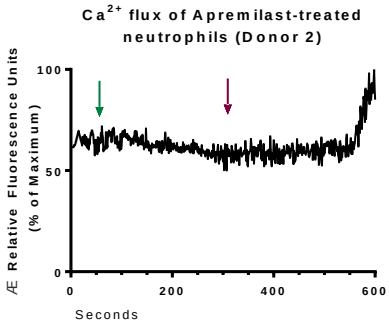

Ca2+ flux of untreated neutrophils
(Donor 2)
Æ Relative Fluorescence Units
(% of Maximum)
100
50
0
0
200
400
600
Seconds

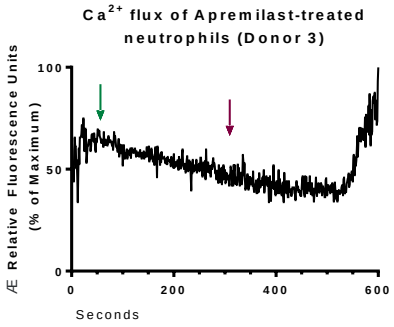

Ca2+ flux of untreated neutrophils
(Donor 3)
Æ Relative Fluorescence Units
(% of Maximum)
100
50
0
0
200
400
600
Seconds


Ca2+ flux of untreated neutrophils
(Donor 4)
Æ Relative Fluorescence Units
(% of Maximum)
100
50
0
0
200
400
500
Seconds

B


Ca2+ flux of Apremilast-treated
neutrophils (Donor 1)
Æ Relative Fluorescence Units
(% of Maximum)
100
50
0
0
200
400
600
Seconds


Ca2+ flux of Apremilast-treated
neutrophils (Donor 2)
Æ Relative Fluorescence Units
(% of Maximum)
100
50
0
0
200
400
600
Seconds


Ca2+ flux of Apremilast-treated
neutrophils (Donor 3)
Æ Relative Fluorescence Units
(% of Maximum)
100
50
0
0
200
400
600
Seconds

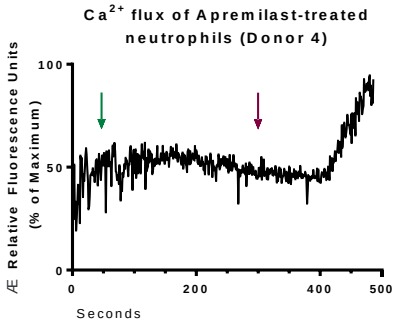

Ca2+ flux of Apremilast-treated
neutrophils (Donor 4)
Æ Relative Fluorescence Units
(% of Maximum)
100
50
0
0
200
400
500
Seconds

Figure 4.14| The *ex vivo* treatment of healthy donor-derived neutrophils with Apremilast, can diminish the cells' intracellular calcium transients in response to CXCL8

Flow-cytometric analysis of the intracellular calcium flux of the intracellular calcium flux of untreated (A) or ex vivo Apremilast-treated (B) neutrophils, derived from 4 healthy donors, in response to 50ng/mL CXCL8 and 2µM Ionomycin (positive control). Following a 2h incubation at 37^{o}C with neutrophil media or neutrophil media + 100µM Apremilast, neutrophils were stained with the Ca^{2+} fluorescence indicator Fluo4 and analysed via FACS. A baseline readout was taken for 50 seconds; 50ng/mL CXCL8 was added at 50 seconds (green arrow); calcium transients were allowed to return to baseline; ionomycin was added at 300 seconds (red arrow) and fluxes were allowed to peak (usually 600 seconds); where the ionomycin peak came at an earlier point (Donor 4) - the FACS analysis was stopped. The data is presented in the form of a graph chart and show the calcium transients of 4 healthy donors: the graph showing the flux of untreated cells is to the left of the graph showing the flux of treated cells of the corresponding patient.

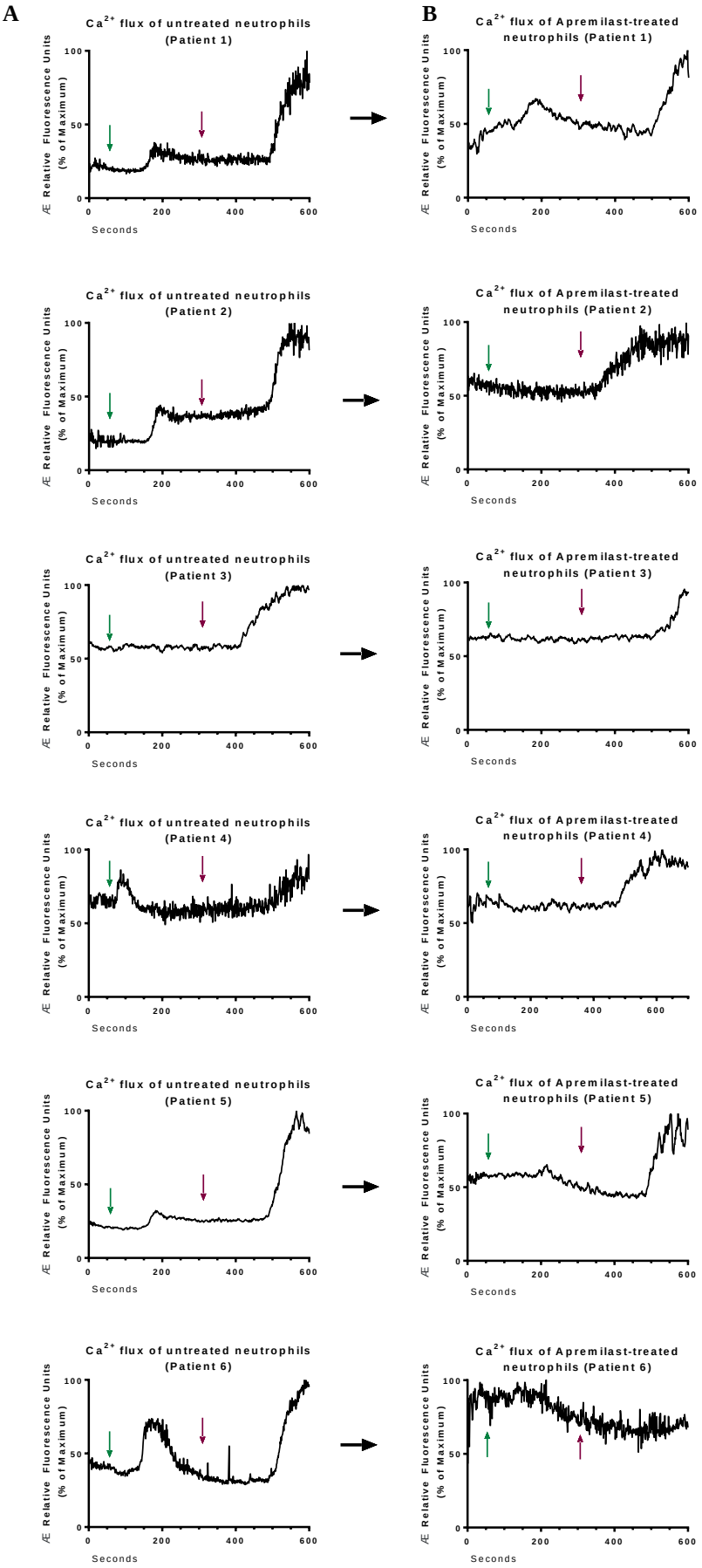

A
B
Ca2+ flux of untreated neutrophils (Patient 1)
Ca2+ flux of Apremilast-treated neutrophils (Patient 1)
Ca2+ flux of untreated neutrophils (Patient 2)
Ca2+ flux of Apremilast-treated neutrophils (Patient 2)
Ca2+ flux of untreated neutrophils (Patient 3)
Ca2+ flux of Apremilast-treated neutrophils (Patient 3)
Ca2+ flux of untreated neutrophils (Patient 4)
Ca2+ flux of Apremilast-treated neutrophils (Patient 4)
Ca2+ flux of untreated neutrophils (Patient 5)
Ca2+ flux of Apremilast-treated neutrophils (Patient 5)
Ca2+ flux of untreated neutrophils (Patient 6)
Ca2+ flux of Apremilast-treated neutrophils (Patient 6)
Æ Relative Fluorescence Units (% of Maximum)
Seconds
0
50
100
200
400
600

Figure 4.15| The *ex vivo* treatment of psoriasis donor-derived neutrophils with Apremilast, can diminish the cells' intracellular calcium transients in response to CXCL8

Flow-cytometric analysis of the intracellular calcium flux of untreated (A) or ex vivo Apremilast-treated (B) neutrophils, derived from 6 patients with psoriasis, in response to 50ng/mL CXCL8 and 2μM Ionomycin (positive control). Following a 2h incubation at 37°C with neutrophil media or neutrophil media + 100μM Apremilast, neutrophils were stained with the Ca^{2+} fluorescence indicator Fluo4 and analysed via FACS. A baseline readout was taken for 50 seconds; 50ng/mL CXCL8 was added at 50 seconds (green arrow); calcium transients were allowed to return to baseline; ionomycin was added at 300 seconds (red arrow) and fluxes were allowed to peak (600s). The data is presented in the form of a graph chart and show the calcium transients of 4 healthy donors: the graph showing the flux of untreated cells is to the left of the graph showing the flux of treated cells of the corresponding patient.

4.5 Summary and conclusions

In this chapter, we investigated the effects of Apremilast on neutrophil migration and influx into psoriasiform skin. This examination was prompted by last chapter's discovery that Apremilast treatment of patients with psoriasis leads to a decrease in neutrophil, but not T-cell, migration. Apremilast is a PDE4 inhibitor which inhibits the production of multiple pro-inflammatory mediators and could therefore inhibit neutrophil migration indirectly[124-126]. From our studies, it appears that high concentrations of Apremilast (100 μM) can directly inhibit the responsiveness of psoriatic neutrophils to CXCL8. This is in conjugation with previous reports that other, less specific, PDE4 inhibitors can affect neutrophil function and migration in the lung[664]. However, prior to drawing final conclusions a cell toxicity assay would need to be run. Interestingly, albeit no significance was recorded, treatment of healthy neutrophils with 0.1μM Apremilast resulted in a non-significant decrease in CXCL8 responsiveness. Contrary to this, 0.1μM Apremilast treatment of psoriatic neutrophils did not result in a similar trend. This could suggest that the migratory responsiveness of psoriatic neutrophils is hard to attenuate with a low Apremilast dose. A similar pattern of migration attenuation was recorded between Apremilast-treated psoriatic and healthy neutrophils; however, the reduction in migration of healthy neutrophils was not statistically significant. This could suggest that Apremilast inhibits chemokine responsiveness and migration regardless of the inflammatory state of a cell. However; it is important to mention that the effects of Apremilast on neutrophil responsiveness to CXCL8 are more prominent in psoriatic cells. This could be explained by the heightened CXCL8-responsiveness of psoriatic neutrophils. As discussed in the introduction of this thesis, psoriasis is a systemic condition, associated with increased levels of multiple pro-inflammatory cytokines[2] (e.g. TNF, CXCL8, IFN, IL-17) which can affect leukocyte activation and responsiveness to different cues. It is, therefore, likely that psoriatic neutrophils become primed by the inflammatory state associated with the disease. We suggest that Apremilast 'resets' the psoriatic neutrophils and allows the cells to respond to CXCL8 in a similar to healthy neutrophils way.

It is thought that PDE4 inhibition can trigger intracellular signalling pathways that inhibit responsiveness to key neutrophil attracting chemokines, such as CXCL8[664,910,911]. Importantly, our findings show that Apremilast does not significantly affect the immediate surface expression of CXCR2 on psoriatic neutrophils. However, a trend which suggests Apremilast-induced downregulation of CXCR2 expression on psoriatic neutrophils was observed. Therefore, we propose that Apremilast-induced signalling pathways that result in decreased CXCL8 responsiveness might not be restricted to chemokine receptor expression (or at least not entirely). Furthermore, CXCL8 binds to more than once receptor and therefore it would be beneficial to

examine the expression of other receptors, such as CXCR1. Interestingly, our data showed that Apremilast can significantly affect the surface expression of CXCR2 on healthy neutrophils. It is possible that we did not see such Apremilast-driven CXCR2 reduction in psoriatic neutrophils due to their pro-inflammatory nature and higher CXCL8 responsiveness. It is important to note, that changes in chemokine receptor expression typically take longer than 2 hours (the time we treated neutrophils with Apremilast for) and could therefore be affected by a longer treatment period[914]. This, however, would be difficult to achieve as neutrophils have a very short lifespan, especially ex vivo, and are rapid to change gene expression signature. No previous studies have investigated the effects of PDE4 inhibition on long-term chemokine receptor expression, perhaps due to the outlined reasons.

Furthermore, it has previously been suggested that direct PDE4 inhibition and subsequent cAMP elevation in neutrophils can lead to a re-sequestration of cytosolic Ca^{2+}[685,687-689]. Importantly, the removal of calcium from neutrophils has also been associated with reduction of neutrophil migratory capabilities[682,685,686]. Here we demonstrate that Apremilast can inhibit neutrophil intracellular Ca^{2+} transients in response to CXCL8, which is in agreement with reports of the activity of other PDE4 inhibitors, such as Roflumilast[664]. It is important to note that regardless of treatment group or donor, there was a delay in the calcium flux of neutrophils in response to the positive control - ionomycin (usually very rapid response). This was due to a technical aspect of the experiment. As this is a real-time experiment and addition of stimulants is done while the flow cytometer is reading the sample, the limitations of the machine have to be taken into account. For example, ionomycin had to be supplied in a larger volume, which would result in a delay of availability to the cells in the experimental tube, hence the delay in measurements as well. This issue did not prevent the success of the experiment, but it could be addressed by utilising a different flow cytometer with better specifications. Further to this, the delay in calcium flux could also be attributed to cell death. However, cell viability assays would need to be run to address this.

To further validate our findings and study the *in vivo* effects of Apremilast treatment on neutrophil function, we utilised the Imiquimod mouse model of psoriasiform inflammation. We demonstrated, via flow-cytometry and histology, that treatment with Apremilast can significantly reduce the influx of $Ly6G^{hi}Ly6C^{int}$ cells into psoriasiform inflamed skin of C56BL/6 mice. It is important to note that flow-cytometry was performed on cells derived from mouse foot skin, whereas histology examinations on back skin. We had to resort to inducing psoriasiform

inflammation in foot skin, as no viable neutrophils could be extracted from back skin. This could be due to multiple reasons related to differences in skin site properties, such as skin thickness and toughness. As skin toughness can vary, multiple factors need to be taken into account when digesting different parts of skin into single cell suspension. Therefore, our attempt to extract neutrophils from back skin of mice could have been unsuccessful due to an inappropriate concentration of digestion enzymes or digestion time. Importantly, we consider this as the most plausible explanation, as neutrophils could be identified in the back skin of Aldara-treated mice via histology. It is important to mention that more specific methods for neutrophil identification in histology samples could be utilised in the future (immunohistochemistry). Further to these findings, Apremilast-treated mice had a lower modified PASI score and epidermal thickness than Apremilast-untreated mice with psoriasiform inflammation and were rescued from a significant weight loss. These data suggest that Apremilast-induced alleviation of psoriasiform inflammation could be, in part, related to reduced neutrophilic skin influx. It is important to mention, that the increase in neutrophil influx in Aldara-treated mice could possibly be attributed to a secondary effect. In order to be able to interrogate that an extra control should be added in future work – Apremilast treatment alone.

Certain technical aspects of the imiquimod mouse model experiments need considered. For example, these experiments were carried out once only, albeit on 5 mice in each group, and the interpretation of the collected data could therefore be more challenging. This was due to the ongoing COVID-19 pandemic. It is important to note that this experimental approach is not a strict representation of Apremilast treatment and assessment (mPASI) of patients with psoriasis. Although Apremilast is not given to patients prior to lesion development, this could highlight the benefits of having an early treatment strategy. Furthermore, the dosing of the mice was challenging, as also reported by other groups, due to difficulties of dissolving Apremilast[917]. Although we reported that Apremilast had a physiological effect on the treated mice, its availability when ingested by the animals need to be carefully considered (e.g. incompletely dissolved drug my not enter circulation). Lastly, it must be taken into account that skin layers can become damaged and pushed closer to each other when histology is performed. Nevertheless, measuring epidermal thickness via histology is a common practice and can be a reliable readout, when performed on good quality slides.

In conclusion, we have determined that Apremilast can directly affect neutrophil responsiveness to CXCL8. Furthermore, we have shown that the loss of CXCL8 responsiveness could be linked to

abrupt intracellular Ca^{2+} regulation, as Ca^{2+}fluxes in response to CXCL8 were also lost. Finally, we demonstrated that Apremilast treatment can alleviate psoriasiform inflammation *in vivo* and this could be related to a reduction of neutrophil influx into psoriasiform skin.

Chapter 5: Sequential order of neutrophil-keratinocyte-T-cell interactions in psoriasis pathogenesis

5.2 Introduction and hypotheses

In the previous chapters, we demonstrated and highlighted the importance of neutrophils, and neutrophil- and T-cell migration to the inflammatory process in psoriasis. However, neutrophils and other leukocytes, such as T-cells, are a part of a considerable network of cells and interactions that determine the course of disease development[11-13]. The key cellular interactions in the early stages of plaque development that lead to aberrant chemokine production, leukocyte recruitment and pro-inflammatory feedback loops, are poorly defined. T-cells and neutrophils are amongst the first cells to arrive in skin during psoriasis plaque development and are known to be involved in cross-talk with keratinocytes[17,707,725]. The resultant functional outcomes of leukocyte-keratinocyte cross-talks are however, poorly understood. Deciphering these cellular interactions will give us a better insight into disease initiation and progression, potentially unmasking novel strategies for future therapy development.

A pivotal interaction in psoriasis pathogenesis, which is likely largely chemokine-dependent, is between T cells and keratinocytes[72,568,825,827]. T helper 17 (Th17) cells and the IL-23/IL-17 axis are now considered central to the development of psoriasis pathology[721,762] (as evidenced by the high efficacy of IL-23/IL-17 targeting drugs (Table 1)). IL-23 is overproduced by e.g. dendritic cells in skin lesions and has been linked to the expansion of Th17 populations[112,740]. This could lead to the augmented production of Th17-related cytokines, such as IL-17 and IL-22, which have a profound effect on the surrounding stromal cells[765,766]. For example, IL-17 and IL-22 can induce local keratinocytes to produce Th17 and neutrophil attracting chemokines, such as CCL20 and CXCL8, respectively. Considering that Th17 cells could have a resident status in the psoriatic skin, this could potentially be an early mechanism of leukocyte recruitment to the plaques. Therefore, it was hypothesised that **skin-targeting CCL20-responsive psoriatic T-cells interact with keratinocytes to orchestrate subsequent neutrophil and T-cell recruitment in a chemokine-dependent manner.**

Further to this, the role of neutrophils and neutrophil interactions with keratinocytes in psoriasis remains poorly understood. Much like T-cells, neutrophils can be found early during plaque development[25,722]. Furthermore, neutrophils are known to express cytokines, which can activate and induce keratinocytes and leukocytes to express pro-inflammatory mediators. For example, neutrophils can store IL-17 in their granules and can produce CXCL8[723,825]. This could allow neutrophils to feed into the IL-17/IL-23 axis and to contribute to T-cell and further neutrophil recruitment. However, relatively little is known about the role of neutrophils in early psoriasis pathogenesis. Therefore, it was hypothesised that **skin targeting CXCL8-responsive psoriatic neutrophils interact with keratinocytes to orchestrate subsequent neutrophil and T-cell**

recruitment in a chemokine-dependent manner. Although, T-cells and neutrophils are present early in plaque development, the sequence of events in terms of cell infiltration and leukocyte interactions with stromal cells, too remains poorly understood.

To test these hypotheses, we established leukocyte-keratinocyte co-cultures. Neutrophils and T-cells were derived from patients with psoriasis and were selected from peripheral blood based on responsiveness to CXCL8 and CCL20, respectively. To enable effective comparison between the effects of healthy control and psoriasis leukocytes on keratinocytes, we co-cultured leukocytes with a limited number of reference primary keratinocytes derived from three healthy controls.

Subsequently, we reasoned that leukocytes responsive to psoriasis-associated chemokines, would be more likely to be recruited to the plaques of patients with psoriasis. The generated *ex vivo* co-culture supernatants were then used as chemoattractants in our sequential migration assay. For example, tissue-culture supernatants from neutrophil-conditioned keratinocytes were used as T-cell chemoattractants and vice versa. The importance of CXCL8 and CCL20 in leukocyte recruitment in psoriasis was highlighted in Chapter 3 of this thesis. Based on this, **we hypothesised that if the leukocyte-conditioned keratinocyte tissue-culture supernatant can induce neutrophil or T-cell migration, CXCL8 or CCL20 could be key supernatant components**. We examined this hypothesis by neutralising chemokines from supernatants, prior to the transmigration assay, and via Luminex. Further to this, we performed qPCR analysis of the leukocyte-conditioned keratinocytes to identify possible pro-inflammatory cytokine/chemokine gene up-regulation. By examining the sequential order of interactions between T-cells, neutrophils and stromal cells, we are hoping to find out what induces epidermal infiltration of cells and leads to plaque development.

5.3 Chapter aims

- To establish stromal-leukocyte co-cultures.
- To determine if T-cell-conditioned keratinocytes are responsible for the recruitment neutrophils or if neutrophil-conditioned keratinocytes are responsible for the recruitment T-cells.
- To Define the differences between the KC secretome, induced by T cells and neutrophils.

5.4 Results

5.4.1 Establishing leukocyte-stromal cell co-cultures

The inflammatory process in psoriasis is dependent on complex cell-to-cell interactions, which culminates in establishing multiple pro-inflammatory axes and enhanced leukocyte recruitment[17,707,725]. Importantly, the formation of pro-inflammatory axes, such as the IL-17/IL-23 axis, could allow the inflammatory process to become self-sustained/self-amplifying, which represents a difficulty when treating psoriasis[27]. Understanding the early cellular interactions that lead to leukocyte recruitment to the skin, could help develop more specific treatments for psoriasis, and possibly at a stage before plaques become established.

In order to study keratinocyte-leukocyte interactions in psoriasis, we aimed to establish keratinocyte co-cultures with neutrophils or T-cells. Before addressing complex questions, such as the impact of leukocytes on keratinocyte chemokine secretome, we wanted to determine whether addition of leukocytes would be detrimental to keratinocyte viability. At the time of T-cell-keratinocyte co-culture optimisation, we were unable to recruit patients with psoriasis for reasons previously outlined. Therefore, these optimisations were completed using T-cells which were isolated from healthy donors and then CD2/CD28-activated. In an initial co-culture set up, we added varying numbers of T-cells (1000, 5000, or 20 000) to keratinocyte cultures. These were primary human keratinocyte cultures, whereby keratinocytes were derived from at least three independent donors. Keratinocytes were added at an initial confluence of 70-80% per well (12 well-plate), allowed to adhere overnight and then exposed to T-cells for 6 hours. It is important to mention that the co-cultures were established in keratinocyte media and no neutrophil (G-CSF) or T-cell (cytokines) growth/stimulating factors were added. Both 1000 (Figure 5.1 B) and 5000 (Figure 5.1 C) activated T-cells were tolerated by the keratinocyte cultures, and no visible apoptosis was induced. Apoptosis was not measured experimentally – only visual inspection took place. Adding 20 000 activated T-cells (Figure 5.1 C) was detrimental to the keratinocyte cultures, as suggested by cell detachment. Attached keratinocytes were not visible at the 24h time point and therefore presumed dead. An identical neutrophil-keratinocyte co-culture experiment was carried out, yielding similar to T-cell-keratinocyte co-culture results. Therefore, it was decided that 5000 leukocytes would be added to keratinocytes, when creating co-cultures in the experiments described in this chapter.

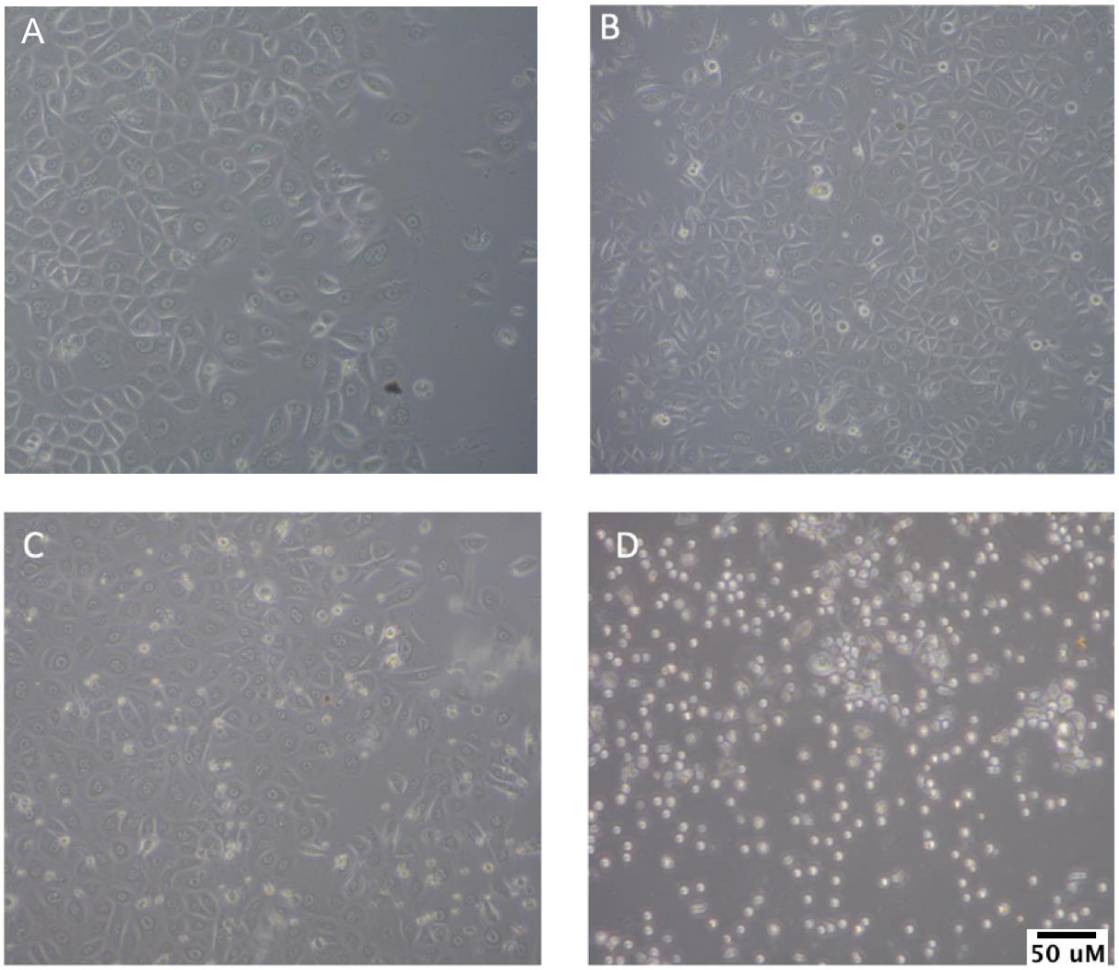


Figure 5.1| High numbers of activated T-cells were detrimental to keratinocyte cultures.

Images of keratinocytes co-cultured with 0 (A), 1K (B), 5K (C) and 20K (D) T- cells. T-cells were derived from healthy donors and were subsequently activated via CD2/CD3/CD28 stimulation. Following a 24h incubation period, the co-cultures were visualised with a light microscope at magnification of 20x and captured by camera. The images are representative of 3 experiments. Scale bar is: 50 microns.

In Chapter 3, we developed an *ex vivo* transmigration assay, which allowed us to investigate the ability of psoriasis-associated chemokines to recruit leukocytes, in an adaptable and reductionist model. We demonstrated that psoriatic neutrophils and T-cells had a heightened migratory responsiveness to CXCL8 and CCL20, respectively. This also suggested for a heightened pro-inflammatory state of leukocytes in question. In this chapter, we used CXCL8 and CCL20 to select peripheral blood-derived neutrophils and T-cells, respectively, for co-culture with keratinocytes. This could potentially allow for the selection of pro-inflammatory leukocytes that would likely be recruited to psoriatic lesions, compared to other circulating PBMCs that are not CCL20 or CXCL8 responsive.

As mentioned earlier, the IL-17/IL-23 pro-inflammatory axis is an important driver of inflammation in psoriasis[27]. Importantly, the main T-cell subtype orchestrator/driver of inflammation in psoriasis, Th17 cells, express high levels of CCR6 (the CCL20 receptor) and are the main producers of IL-17[51,55]. IL-17 could induce CCL20 production by keratinocytes[62]. It is believed, that the CCR6-CCL20 chemokine/chemokine receptor pair is the main driver of Th17 recruitment and retention in the psoriatic lesion[831]. Therefore, the Th17-induced production of CCL20 by keratinocytes could feed into the, already fuelled by neutrophils and T-cells, IL-17/IL-23 axis, though this has not been previously shown. Selecting T-cells on the basis of their CCL20-responsiveness could allow us to further interrogate this pro-inflammatory axis and address its relevance to neutrophil recruitment.

Before culturing the CCL20-responsive T-cells with keratinocytes, we wanted to study T-cell cell surface and intracellular molecular expression. This would allow us to better understand the phenotype of CCL20-responsive T-cells. In order to do this, T-cells were isolated from the peripheral blood of patients with psoriasis and migrated towards CCL20. Cells were then stained with antibodies against CD3, CD4, CCR6 and RORγT. Due to a clash in fluorophore antibody colours the stains had to be performed in duplicates. The cells were divided into two separate tubes, one tube was stained with antibodies against CD3 and CCR6, whereas the one against CD4 and RORγT. The flow cytometric analysis showed that at least 90% of the cells in every sample had high expression of all of the markers tested (Figure 5.2). This suggested that the CCL20-responsive T-cells were of a Th17 phenotype.

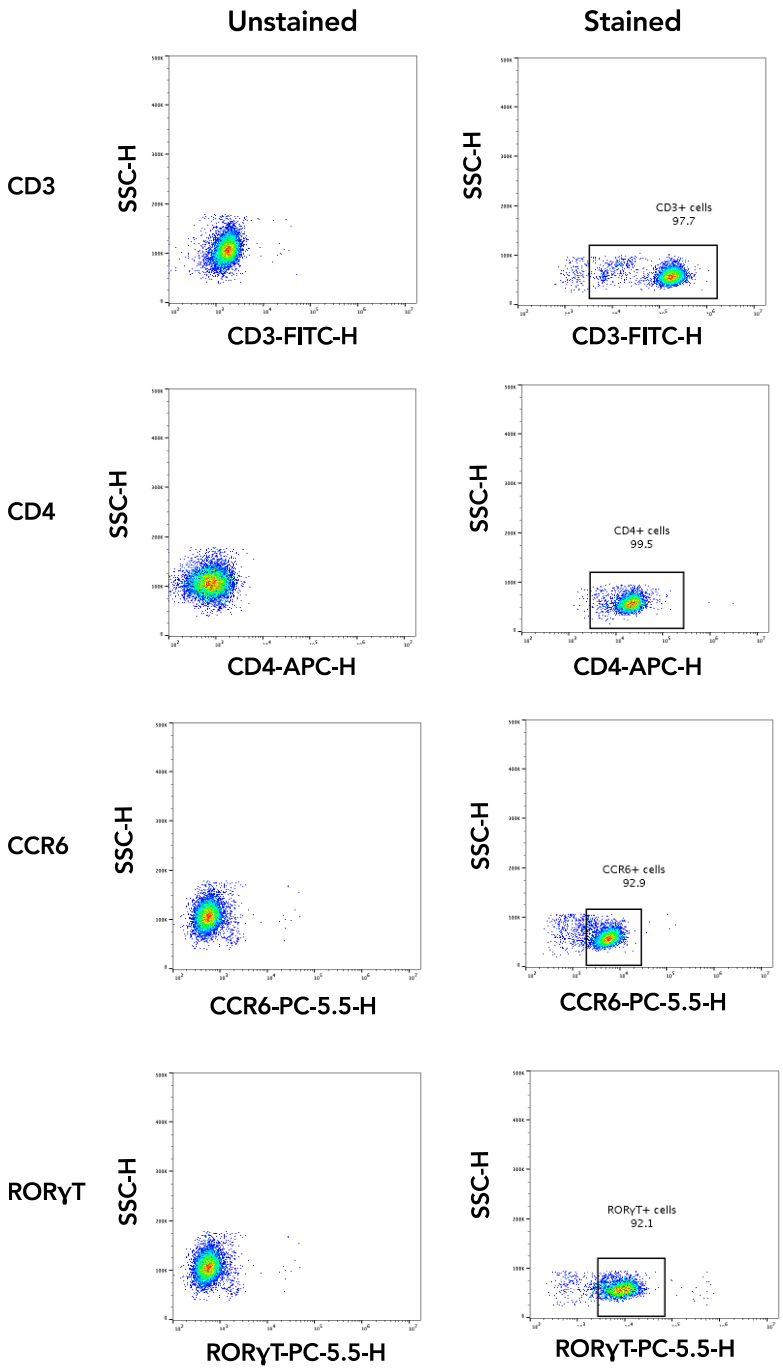


Figure 5.2| CCL20-responsive psoriatic T-cells expressed CD3, CD4, CCR6 and RORγT

Flow-cytometric analysis showing the expression of cell surface molecules, such as CD3, CD4, and CCR6, and intracellular transcription factor RORγT. T-cells were isolated from the peripheral blood of patients with psoriasis and migrated towards CCL20 for 1.5h. Cells were then stained with fluorophore-conjugated antibodies against the aforementioned molecules. The stains were applied in doubles, whereby one part of the cells was stained with CD3 and CCR6, and another part with CD4 and RORγT. Cells were permeabilised and fixed prior to RORγT staining. Data representative of 3 experiments.

5.4.2 The role of T-cells and neutrophils in the production of leukocyte-attracting factors by keratinocytes

Chemokines play an important role in the recruitment of leukocytes and therefore, possibly, the initiation and sustainment of inflammation in psoriasis. However, it remains unclear what early interactions lead to elevated chemokine production and subsequent leukocyte recruitment. Even less clear is the sequence of migratory events; **whether the outcome of T-cell-stromal cell interactions is neutrophil recruitment, or vice versa.**

In order to investigate this, a series of leukocyte-conditioned keratinocyte tissue-culture supernatants were generated and then used as chemoattractants (Figure 5.3). For example, 5000 CXCL8-responsive healthy or psoriatic neutrophils were co-cultured with keratinocytes for a period of 24 hours. The resultant supernatant is referred to as 'KC + Neutrophils' in the figures below. Neutrophils were then removed, keratinocytes washed, and fresh supernatant was given to these keratinocytes for a further 24-hour period. The resultant supernatant is referred to as 'KC post neutrophil removal' in the figures below. The supernatant from the first 24 hours contained factors produced from both neutrophils and keratinocytes, whereas the second supernatant contained the secretome of neutrophil-conditioned keratinocytes only. These supernatants were then used as chemoattractants for T-cells and neutrophils in transmigration assays. This schedule was repeated with T-cell-keratinocyte co-cultures and subsequent transmigration was carried out with the resultant supernatants. A graphical representation of the schedule can be found below (Figure 5.2a).

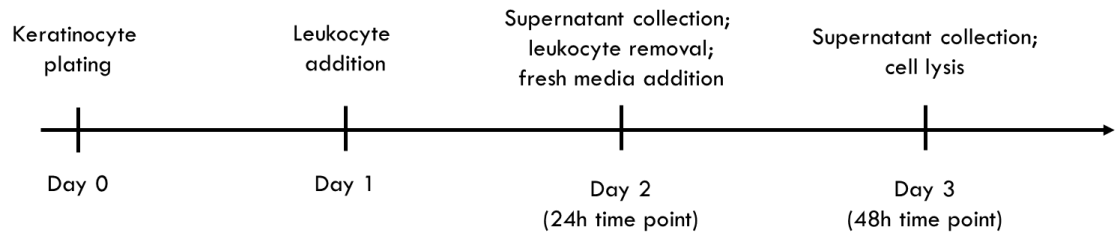


Figure 5.2a| Graphical representation of the schedule followed when generating tissue culture supernatants for sequential migration studies

Before starting the sequential transmigration assays, we wanted to determine the optimal dilution of the co-culture supernatants, when used as chemoattractants. Oversaturation with chemokines can prevent optimal migration. At the time of this optimisation we were unable to recruit patients with psoriasis, therefore we had to diversify our methodologies. T-cells and neutrophils were derived from healthy donors and were then activated via CD3/CD28 and Lipopolysaccharide (LPS)-stimulation, respectively. The activated cells were then cultured with keratinocytes, as per the schedule described above and disclosed in Figure 5.3. The resultant supernatants were diluted at a ratio of 1:1, 1:5, or 1:25 with migration media and were used as chemoattractants. T-cell-conditioned keratinocyte supernatants were used as a chemoattractant for LPS-activated neutrophils, whereas neutrophil-conditioned keratinocyte supernatants as a chemoattractant for CD3/CD28-activated T-cells.

We found that 1:1 diluted T-cell-conditioned keratinocyte supernatant was able to induce significant migration of LPS-activated neutrophils, whereas lower dilutions of the supernatant could not (Figure 5.3 A). Neutrophil-conditioned keratinocyte supernatant could not attract activated T-cells at any dilution (Figure 5.3 B). According to these results, and to keep the experiments consistent, we used a dilution of 1:1 for all type of supernatants in the experiments described in this chapter.

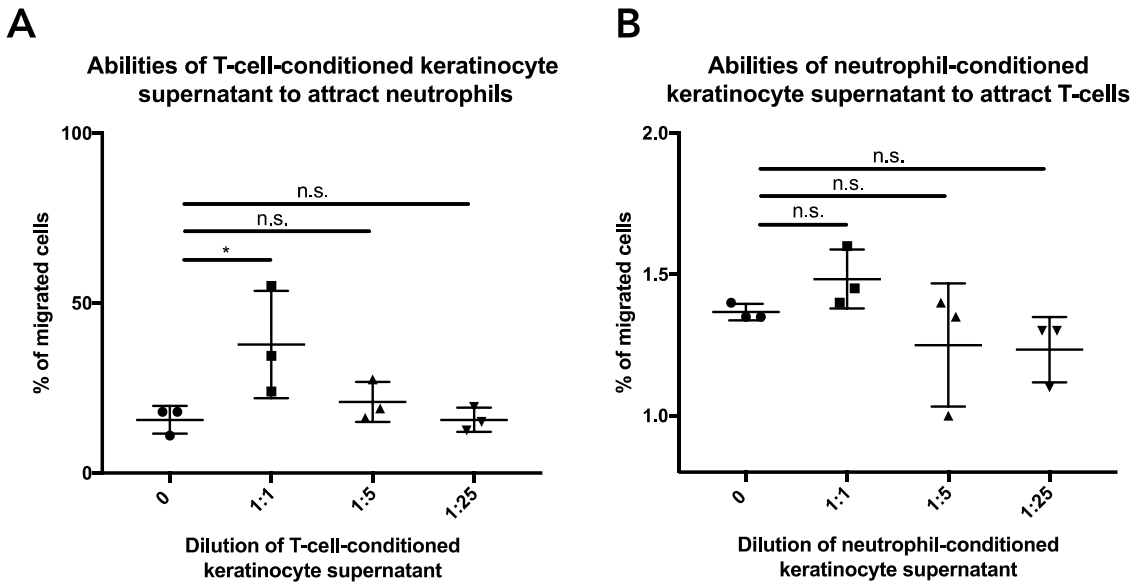


Figure 5.3| 1:1 dilution of conditioned keratinocyte supernatant to migration media was best suited for sequential transmigration experiments

*Transwell analysis of the chemoattractive abilities of T-cell-conditioned (A) and neutrophil-conditioned (B) keratinocyte supernatant at different dilutions. Three different supernatants to migration media dilutions were tested in this experiment: 1:1; 1:5; 1:25. 0 is media only, no supernatant. LPS-activated healthy donor-derived neutrophils were used to assess the chemoattractive abilities of T-cell-conditioned keratinocyte supernatant (A). CD2/CD3/CD28-activated healthy donor-derived T-cells were used to assess the chemoattractive abilities of neutrophil-conditioned keratinocyte supernatant (B). Following a 1h (A) or 1.5h (B) incubation period in transwell chambers, migrated cells were collected and counted. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (n=3 samples) (One-way ANOVA test; *p<0.05).*

5.4.2.1 The role of T-cells in the production of neutrophil-attracting factors by Keratinocytes

Next, we wanted to determine the ability of T-cells to stimulate the production of neutrophil-attracting factors by keratinocytes. For the purpose, T-cells were isolated from the peripheral blood of patients with psoriasis and healthy donors and selected based on CCL20-responsiveness. CCL20-responsive T-cells were co-cultured with keratinocytes, according to the schedule described in Figure 5.2a, and resultant supernatants were used as chemoattractants for neutrophils. Supernatants resulting from an initial 24h co-culture of keratinocytes with psoriatic T-cells ('KC+T-cells' in figures), did not induce a significant transmigration of psoriatic neutrophils (Figure 5.4 A). However, T-cells were removed from the co-cultures and keratinocytes were given fresh media for a further 24h period. The resulting supernatants ('KC post T-cell removal' in figures) could induce a significant increase in the migration of the same neutrophils (Figure 5.4 A). This suggested that the induction of the production of chemoattractive factors by keratinocytes is time-dependent. Conversely, supernatants derived from keratinocyte and healthy donor-derived T-cell co-cultures did not induce transmigration of psoriatic neutrophils at any time point tested (Figure 5.4 B). Importantly, none of the supernatants outlined above could induce significant transmigration of healthy neutrophils (Figure 5.5). These findings, again, suggest for the heightened pro-inflammatory state of psoriatic leukocytes.

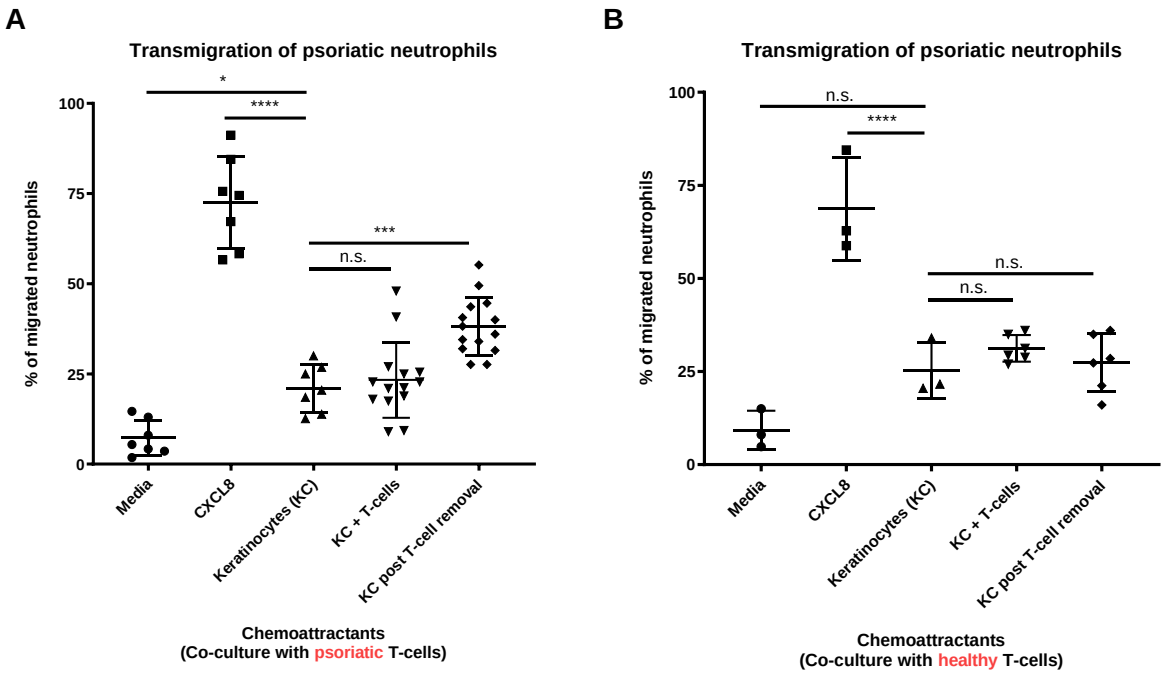


Figure 5.4| Supernatants of keratinocytes conditioned by psoriatic, but not healthy, T-cells induced migration of psoriatic neutrophils

*Transwell analysis of the chemoattractive abilities of supernatants of keratinocytes conditioned by T-cells derived from patients with psoriasis (A) or healthy donors (B). Psoriatic neutrophils were used to assess the chemoattractive abilities of the T-cell-conditioned keratinocyte supernatants. Following a 1h incubation period in transwell chambers, migrated neutrophils were collected and counted. Keratinocytes for co-culture were derived from at least two different donors. Only CCL20-responsive T-cells were used for co-culture. In (A) T-cells were derived from 15 different donors, therefore 15 different T-cell conditioned supernatants were generated and utilised. The neutrophils of 7 of the donors were migrated towards two different supernatants. The neutrophils of 1 of the donors were migrated towards one supernatant. In (B) T-cells were derived from 6 different donors, therefore 6 different T-cell conditioned supernatants were generated and utilised. The neutrophils of 3 of the donors were migrated towards two different supernatants. Chemoattractants from left to right are: 1. Media - transmigration media only (negative control); 2. CXCL8 (positive control); 3. Keratinocyte - Supernatant of 24-48h Keratinocyte cultures; 4. KC+T-cells - Supernatant of 24h Keratinocyte and T-cell co-cultures; 5. KC post T-cell removal - Supernatant of 24h Keratinocyte cultures, but prior to this the keratinocytes were co-cultured with T-cells for 24h (KCs from 4). Data are presented as dot plots, with each dot representing a different biological sample migration, with the Mean+SD (One-way ANOVA test; *p<0.05; ***p<0.001; ****p<0.0001; n.s. = non-significant).*

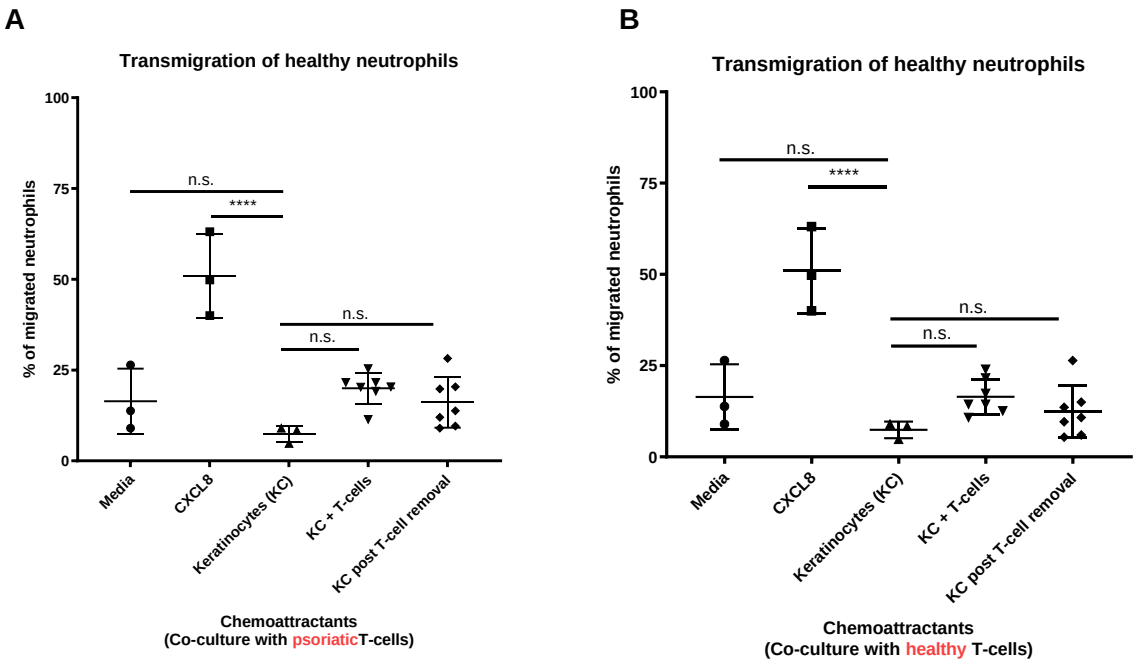


Figure 5.5| Supernatants of keratinocytes conditioned by psoriatic or healthy T-cells did not induce migration of healthy neutrophils

*Transwell analysis of the chemoattractive abilities of supernatants of keratinocytes conditioned by T-cells derived from patients with psoriasis (A) or healthy donors (B). Healthy donor-derived neutrophils were used to assess the chemoattractive abilities of the T-cell-conditioned keratinocyte supernatants. Following a 1h incubation period in transwell chambers, migrated neutrophils were collected and counted. Keratinocytes for co-culture were derived from at least two different donors. Only CCL20-responsive T-cells were used for co-culture. In (A) and (B) leukocytes for co-culture were derived from 7 different donors, therefore 7 different T-cell conditioned supernatants were generated and utilised. The neutrophils of each donor (3 donors in total) were migrated towards at least two different supernatants. Chemoattractants from left to right are: 1. Media - transmigration media only (negative control); 2. CXCL8 (positive control); 3. Keratinocyte - Supernatant of 24-48h Keratinocyte cultures; 4. KC+T-cells - Supernatant of 24h Keratinocyte and T-cell co-cultures; 5. KC post T-cell removal - Supernatant of 24h Keratinocyte cultures, but prior to this the keratinocytes were co-cultured with T-cells for 24h (KCs from 4). Data are presented as dot plots, with each dot representing a different biological sample migration, with the Mean+SD (One-way ANOVA test; ****p<0.0001; n.s. = non-significant).*

In this section, we demonstrated that psoriatic T-cells can induce keratinocytes to produce factors, which can cause neutrophil transmigration. We demonstrated that this is, in fact, characteristic of CCL20-responsive psoriatic T-cells and was time-dependent. Further to this, we suggest that the increase in neutrophil migratory potential is not only present but could be an important feature of cell interaction-induced leukocyte recruitment to the skin.

5.4.2.2 The role of total CD4+ T-cells in the production of neutrophil attracting factors by keratinocytes

We have thus far investigated the importance of psoriatic T-cells in the T-cell-keratinocyte cross-talk cascade, which results in neutrophil recruitment. We wanted to better understand if the effects exerted by T-cells on keratinocytes were related to T-cells being selected on the basis of CCL20-responsiveness. Based on the finding that >99% of the CCL20-responsive T-cells were CD4+ (Figure 5.2), we isolated total CD4+ T-cells from the peripheral blood of patients with psoriasis. These CD4+ T-cells were not selected on the basis of CCL20-responsiveness and were directly co-cultured with keratinocytes, in accordance with the schedule in Figure 5.2a. Hereby, we report that supernatants from CD4+T-cell-keratinocyte co-cultures did not induce a significant increase in the migration of psoriatic neutrophils (Figure 5.6). These data suggest that for T-cells to be able to induce keratinocytes to produce neutrophil attracting factors, T-cells need to be of a particular CCL20-responsive phenotype (e.g. Th17). Another possibility is that the population of total CD4+ T-cell may contain cells which can exert suppressive functions and therefore inhibit keratinocyte stimulation. Regulatory CD4+ T-cells are known to have such functions but their presence and function in the psoriasis plaques has been shown to be very limited.

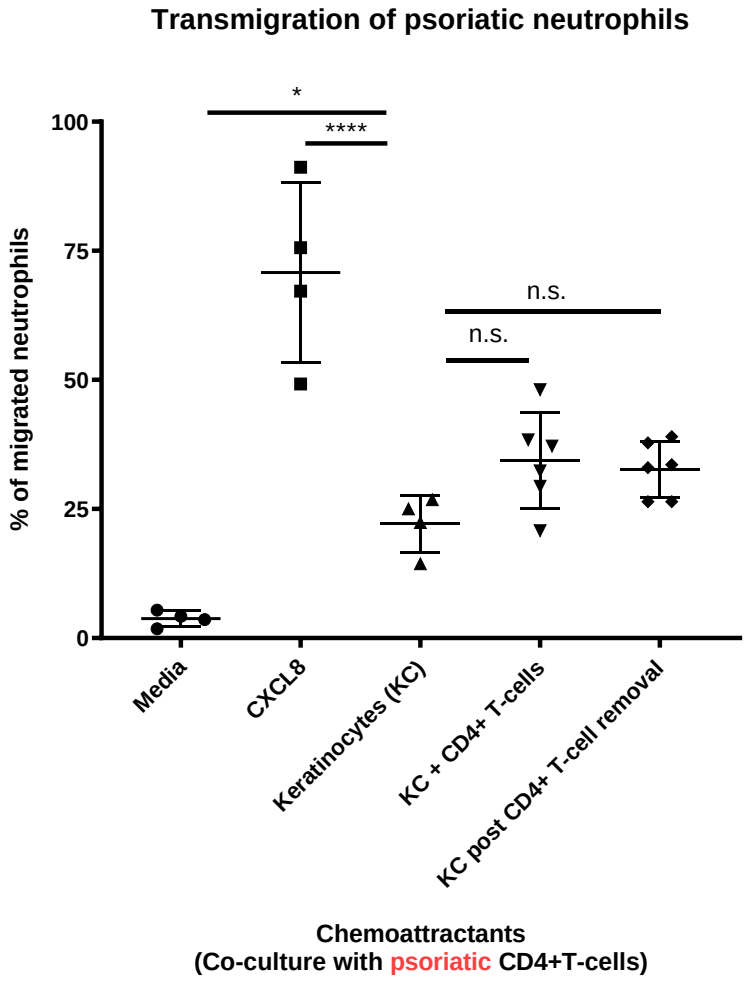


Figure 5.6| Supernatants of keratinocytes conditioned by psoriatic CD4+ T-cells did not induce migration of psoriatic neutrophils

*Transwell analysis of the chemoattractive abilities of supernatants of keratinocytes conditioned by psoriatic CD4+T-cells. Psoriatic neutrophils were used to assess the chemoattractive abilities of the CD4+T-cell-conditioned keratinocyte supernatants. Following a 1h incubation period in transwell chambers, migrated neutrophils were collected and counted. Keratinocytes for co-culture were derived from at least two different donors. CD4+T-cells were not selected based on CCL20-responsiveness. CD4+T-cells for co-culture were derived from 4 different donors, therefore 4 different neutrophil-conditioned supernatants were generated and utilised. The neutrophils of each donor (4 donors in total) were migrated towards 1 to 3 different supernatants. Chemoattractants from left to right are: 1. Media - transmigration media only (negative control); 2. CXCL8 (positive control);3. Keratinocyte - Supernatant of 24-48h Keratinocyte cultures; 4. KC+CD4+T-cells - Supernatant of 24h Keratinocyte and CD4+T-cell co-cultures; 5. KC post CD4+T-cell removal - Supernatant of 24h Keratinocyte cultures, but prior to this the keratinocytes were co-cultured with CD4+T-cells for 24h (KCs from 4). Data are presented as dot plots, with each dot representing a different biological sample migration, with the Mean+SD (One-way ANOVA test; **p<0.01; n.s. = non-significant).*

5.4.2.3 The role of Neutrophils in the production of neutrophil-attracting factors by Keratinocytes

In this subchapter, we wanted to investigate the possible existence of a pro-inflammatory loop that could lead to the auto-amplification of neutrophil recruitment. To do this, we utilised the supernatants resulting from psoriatic neutrophil and keratinocyte co-culture and assessed if they were chemoattractant for neutrophils. Hereby, we report that such dependency was not noted using our experimental model. Neutrophils derived from patients with psoriasis (Figure 5.7 A) and healthy donors (Figure 5.7 B) did not show a significant increase of migration towards supernatants from psoriatic neutrophil-keratinocyte co-cultures. Together, combined with our findings above, this suggests that T-cells, rather than neutrophils, are more important for the induction of keratinocyte-dependent production of neutrophil attracting factors.

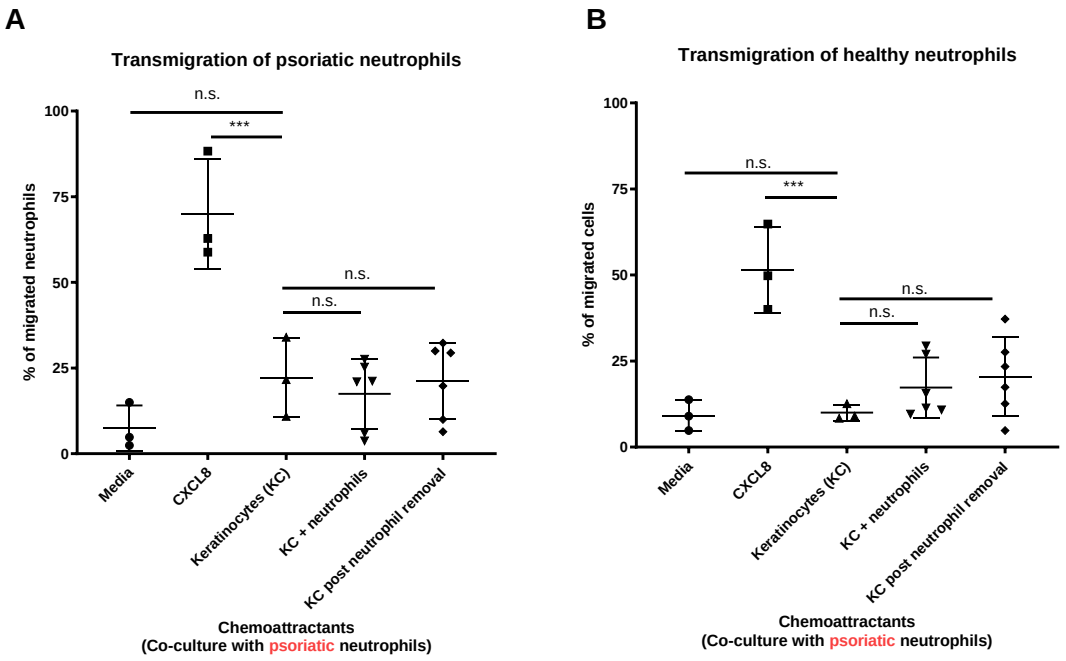


Figure 5.7| Supernatants of keratinocytes conditioned by healthy or psoriatic neutrophils did not induce migration of neutrophils (no auto-amplification occurs)

*Transwell analysis of the chemoattractive abilities of supernatants of keratinocytes conditioned by neutrophils derived from patients with psoriasis (A) or healthy donors (B). Psoriatic (A) and healthy (B) neutrophils were used to assess the chemoattractive abilities of the neutrophil-conditioned keratinocyte supernatants. Following a 1h incubation period in transwell chambers, migrated neutrophils were collected and counted. Keratinocytes for co-culture were derived from at least two different donors. Only CXCL8-responsive neutrophils were used for co-culture. In (A) and (B) leukocytes for co-culture were derived from 6 different donors, therefore 6 different neutrophil-conditioned supernatants were generated and utilised. The neutrophils of each donor (3 donors in total) were migrated towards two different supernatants. Chemoattractants from left to right are: 1. Media - transmigration media only (negative control); 2. CXCL8 (positive control); 3. Keratinocyte - Supernatant of 24-48h Keratinocyte cultures; 4. KC+T-cells - Supernatant of 24h Keratinocyte and T-cell co-cultures; 5. KC post T-cell removal - Supernatant of 24h Keratinocyte cultures, but prior to this the keratinocytes were co-cultured with T-cells for 24h (KCs from 4). Data are presented as dot plots, with each dot representing a different biological sample migration, with the Mean+SD (One-way ANOVA test; ***p<0.001; n.s. = non-significant).*

5.4.2.4 The role of Neutrophils in the production of T-cell-attracting factors by keratinocytes

Another important aim of this chapter was to investigate the effect of neutrophils on the ability of keratinocytes to produce T-cell attracting factors. For the purpose, neutrophils were isolated from the peripheral blood of patients with psoriasis and healthy donors and selected based on CXCL8-responsiveness. CXCL8-responsive neutrophils were co-cultured with keratinocytes and resultant supernatants were used as T-cell chemoattractants. Here we show that none of the supernatants from keratinocytes co-cultured with psoriatic (Figure 5.8 A) or healthy (Figure 5.8 B) neutrophils could induce the transmigration of psoriatic T-cells (Figure 5.8). This was also valid for T-cells that were derived from healthy donors – no significant migration of T cells to any neutrophil-conditioned keratinocyte supernatants occurred (Figure 5.9). Therefore, the cumulative data from chapter 5.4.2 suggest that T-cells, rather than neutrophils, could be more important during the initiation stages, of lesion development, that lead to a heightened leukocyte influx. Further to this and on the basis, that only CCL20-responsive T-cells were utilised, we propose the existence of a pro-inflammatory axis between Th17-associated cytokines-Keratinocytes and neutrophil recruitment.

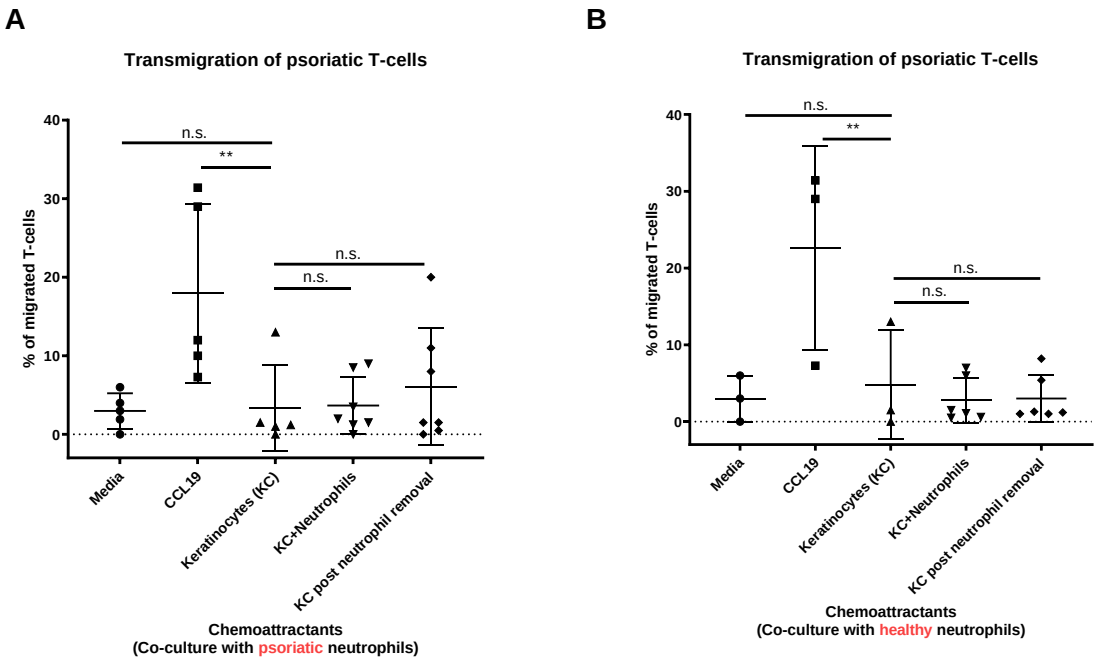


Figure 5.8| Supernatants of keratinocytes conditioned by healthy or psoriatic neutrophils did not induce migration of psoriatic T-cells

*Transwell analysis of the chemoattractive abilities of supernatants of keratinocytes conditioned by neutrophils derived from patients with psoriasis (A) or healthy donors (B). Psoriatic T-cells were used to assess the chemoattractive abilities of the neutrophil-conditioned keratinocyte supernatants. Following a 1h incubation period in transwell chambers, migrated neutrophils were collected and counted. Keratinocytes for co-culture were derived from at least two different donors. Only CXCL8-responsive neutrophils were used for co-culture. In (A) leukocytes for co-culture were derived from 7 different donors, therefore 7 different neutrophil-conditioned supernatants were generated and utilised. The neutrophils of each donor (5 donors in total) were migrated towards 1 to 3 different supernatants. In (B) leukocytes for co-culture were derived from 6 different donors, therefore 6 different neutrophil-conditioned supernatants were generated and utilised. The neutrophils of each donor (3 donors in total) were migrated towards 2 different supernatants. Chemoattractants from left to right are: 1. Media - transmigration media only (negative control); 2. CXCL8 (positive control);3. Keratinocyte - Supernatant of 24-48h Keratinocyte cultures; 4. KC+T-cells - Supernatant of 24h Keratinocyte and T-cell co-cultures; 5. KC post T-cell removal - Supernatant of 24h Keratinocyte cultures, but prior to this the keratinocytes were co-cultured with T-cells for 24h (KCs from 4). Data are presented as dot plots, with each dot representing a different biological sample migration, with the Mean+SD (One-way ANOVA test; **p<0.01; n.s. = non-significant).*

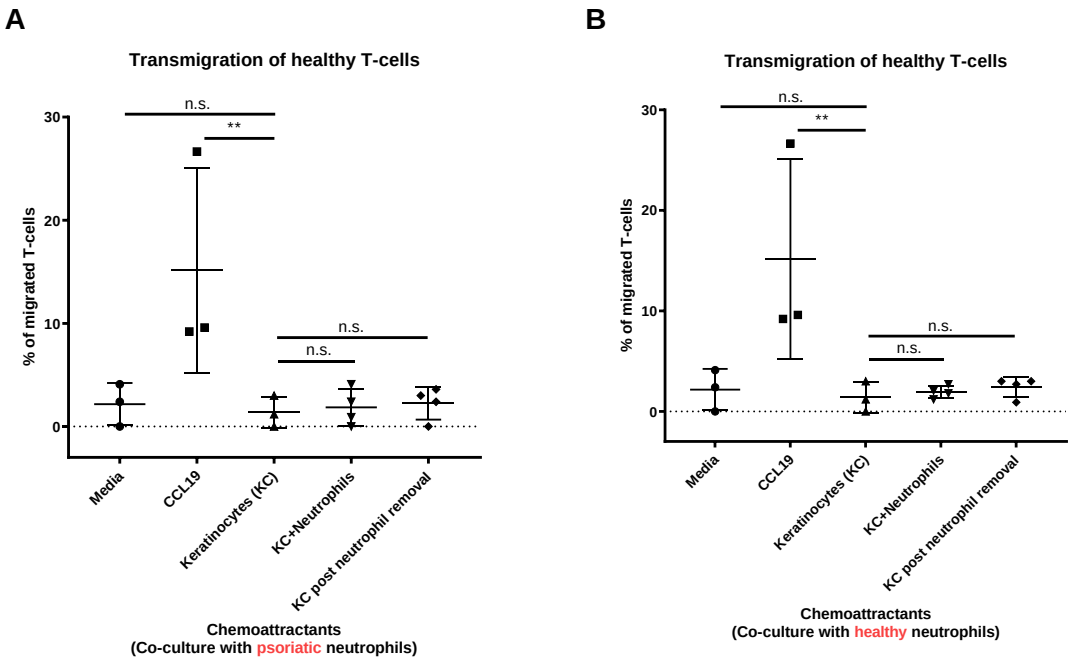


Figure 5.9| Supernatants of keratinocytes conditioned by healthy or psoriatic neutrophils did not induce migration of healthy T-cells

*Transwell analysis of the chemoattractive abilities of supernatants of keratinocytes conditioned by neutrophils derived from patients with psoriasis (A) or healthy donors (B). Healthy donor-derived T-cells were used to assess the chemoattractive abilities of the neutrophil-conditioned keratinocyte supernatants. Following a 1h incubation period in transwell chambers, migrated neutrophils were collected and counted. Keratinocytes for co-culture were derived from at least two different donors. Only CXCL8-responsive neutrophils were used for co-culture. In (A) and (B) leukocytes for co-culture were derived from 4 different donors, therefore 4 different neutrophil-conditioned supernatants were generated and utilised. The neutrophils of each donor (3 donors in total) were migrated towards 1 to 2 different supernatants. Chemoattractants from left to right are: 1. Media - transmigration media only (negative control); 2. CXCL8 (positive control);3. Keratinocyte - Supernatant of 24-48h Keratinocyte cultures; 4. KC+T-cells - Supernatant of 24h Keratinocyte and T-cell co-cultures; 5. KC post T-cell removal - Supernatant of 24h Keratinocyte cultures, but prior to this the keratinocytes were co-cultured with T-cells for 24h (KCs from 4). Data are presented as dot plots, with each dot representing a different biological sample migration, with the Mean+SD (One-way ANOVA test; **p<0.01; n.s. = non-significant)*

5.4.3 Pro-inflammatory cytokines and chemokines in supernatants derived from leukocyte-keratinocyte co-cultures

5.4.3.1 Luminex analysis of pro-inflammatory cytokine and chemokine levels in supernatants derived from leukocyte-keratinocyte co-cultures

Following the functional and gene expression studies, we wanted to examine the protein levels of chemokines and cytokines of interest in leukocyte-keratinocyte supernatants. This could help identify the key chemokine or chemokines, within the supernatants, responsible for the increase in neutrophil migration. The molecular neutrophil and T-cell-attracting and pro-/anti-inflammatory constituents of the generated and studied supernatants was investigated via Luminex. 11 different analytes were examined, these being CXCL8, CCL2, CCL20, CXCL2, IL-17, TNF, CCL17, CXCL1, CXCL10, IL-12, IL-36. All of the supernatants studied and shown in the data above were included in this analysis. Further to this, additional supernatants, generated later during the project, were also included. Only supernatants from keratinocytes co-cultured with psoriatic and healthy cells were examined via Luminex. The baseline level of analyte production by keratinocytes is shown in Figure 5.11. The baseline keratinocyte production values are also included in all of the subfigures to allow appropriate comparisons. Among all of the analytes examined, CXCL8, CCL20, CXCL2, and CXCL1 were present at the highest concentrations (relative to the remainder of the analytes). Our data showed that leukocytes derived from healthy donors were not able to induce an increase in production of any of the examined analytes, except for IL-12, by keratinocytes (Figure 5.12). A significant increase in CXCL8, CCL20, IL-17, TNF, IL-12, and IL-36 levels was noted in supernatants derived from leukocytes co-cultured with keratinocytes (24h timepoint). The production of the aforementioned molecules was not significantly increased following the removal of the leukocytes, in which only KC were present (48h timepoint). More specifically, T-cells were responsible for the increase in IL-17, TNF production; neutrophil presence was linked to higher IL-36 levels; both cell types prompted high CXCL8, CCL20, and IL-12 production when compared to baseline production of the aforementioned molecules by keratinocytes.

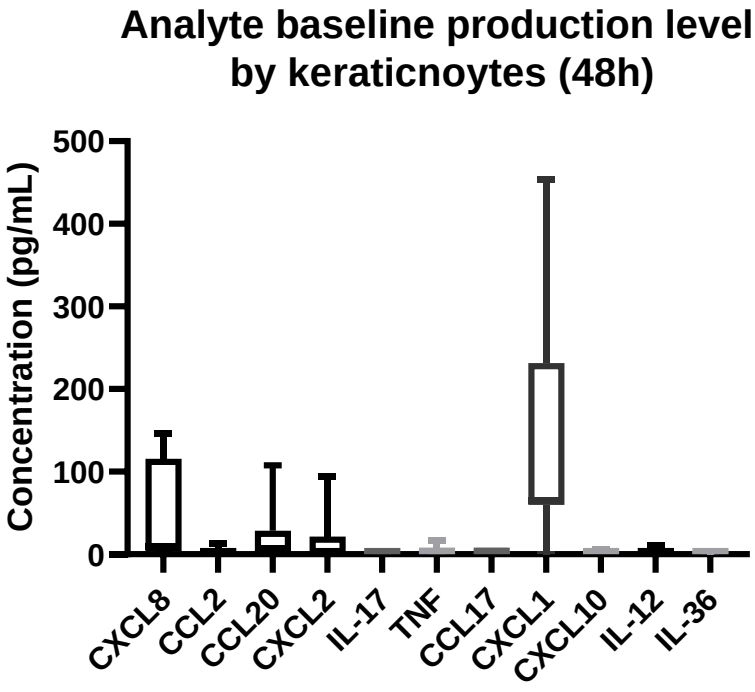
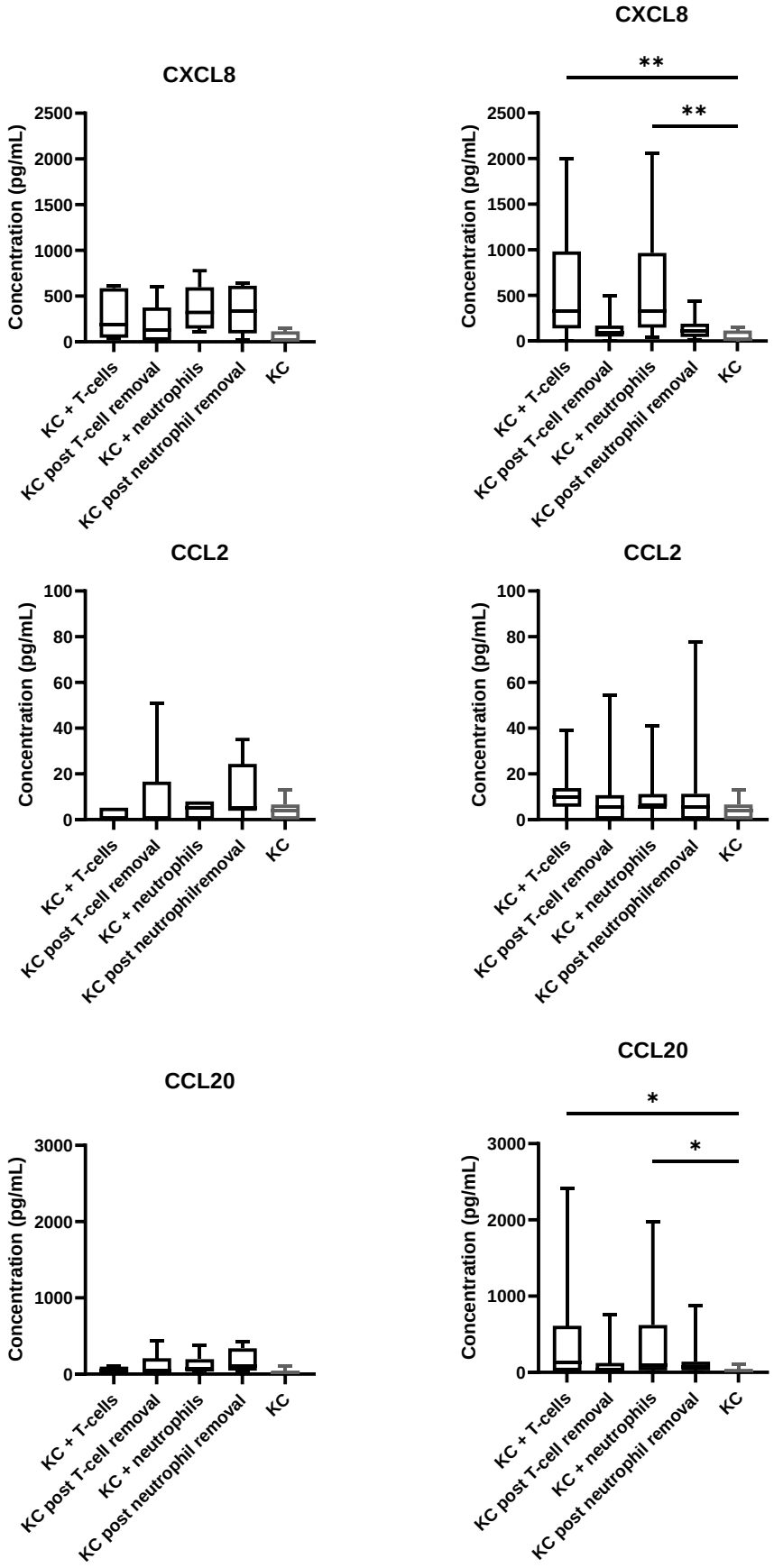


Figure 5.10| The baseline production level of CXCL8, CCL2, CCL20, CXCL12, IL-17, TNF, CCL17, CXCL1, CXCL10, IL-12, and IL-36 by primary keratinocytes.

A graph showing luminex analysis of CXCL8, CCL2, CCL20, CXCL12, IL-17, TNF, CCL17, CXCL1, CXCL10, IL-12, and IL-36 production levels by keratinocytes. Prior to supernatant collection, keratinocytes were cultured for 48h (media change and PBS wash at the 24h time point). Data are presented as box and whiskers plots with the Mean+SD (n= 10) (One-way ANOVA test).

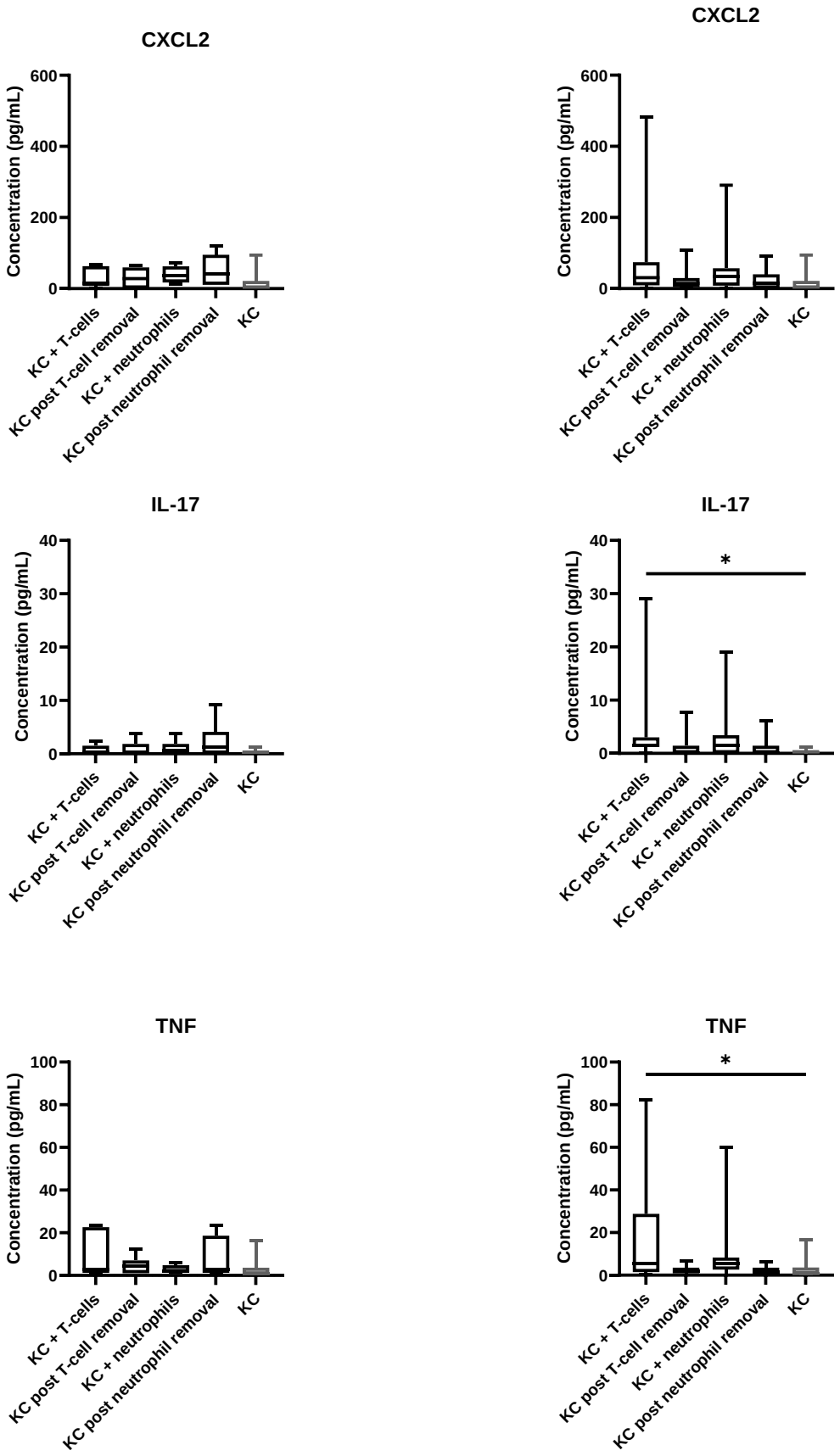
A Healthy non-psoriasis controls

B Patients with psoriasis



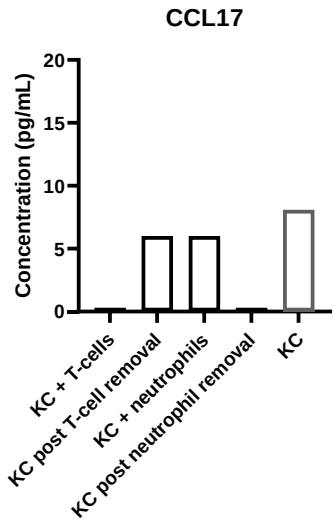
A Healthy non-psoriasis controls

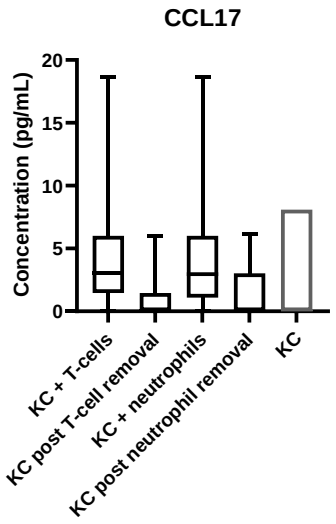
B Patients with psoriasis

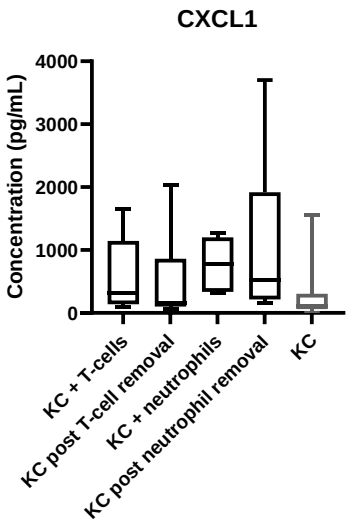


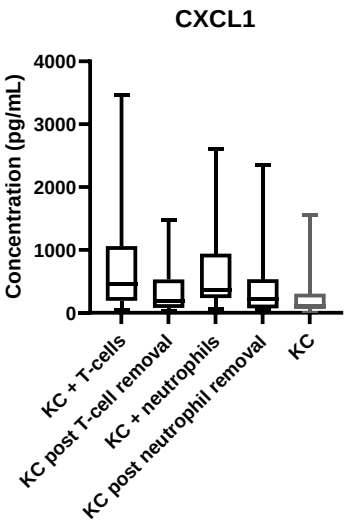
A Healthy non-psoriasis controls

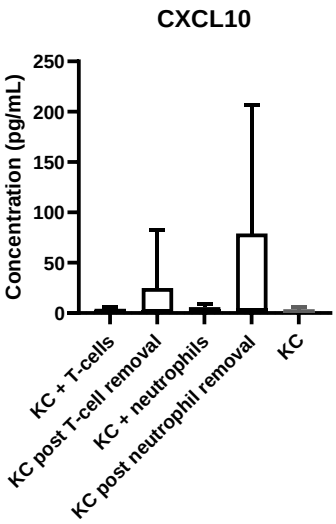
B Patients with psoriasis

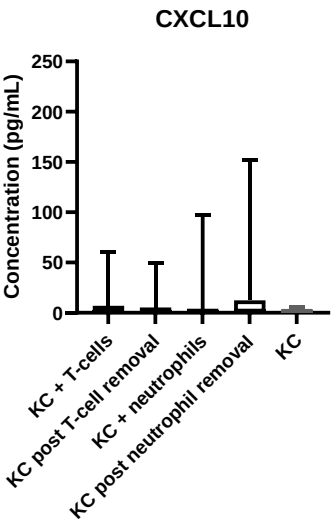












A Healthy non-psoriasis controls

B Patients with psoriasis

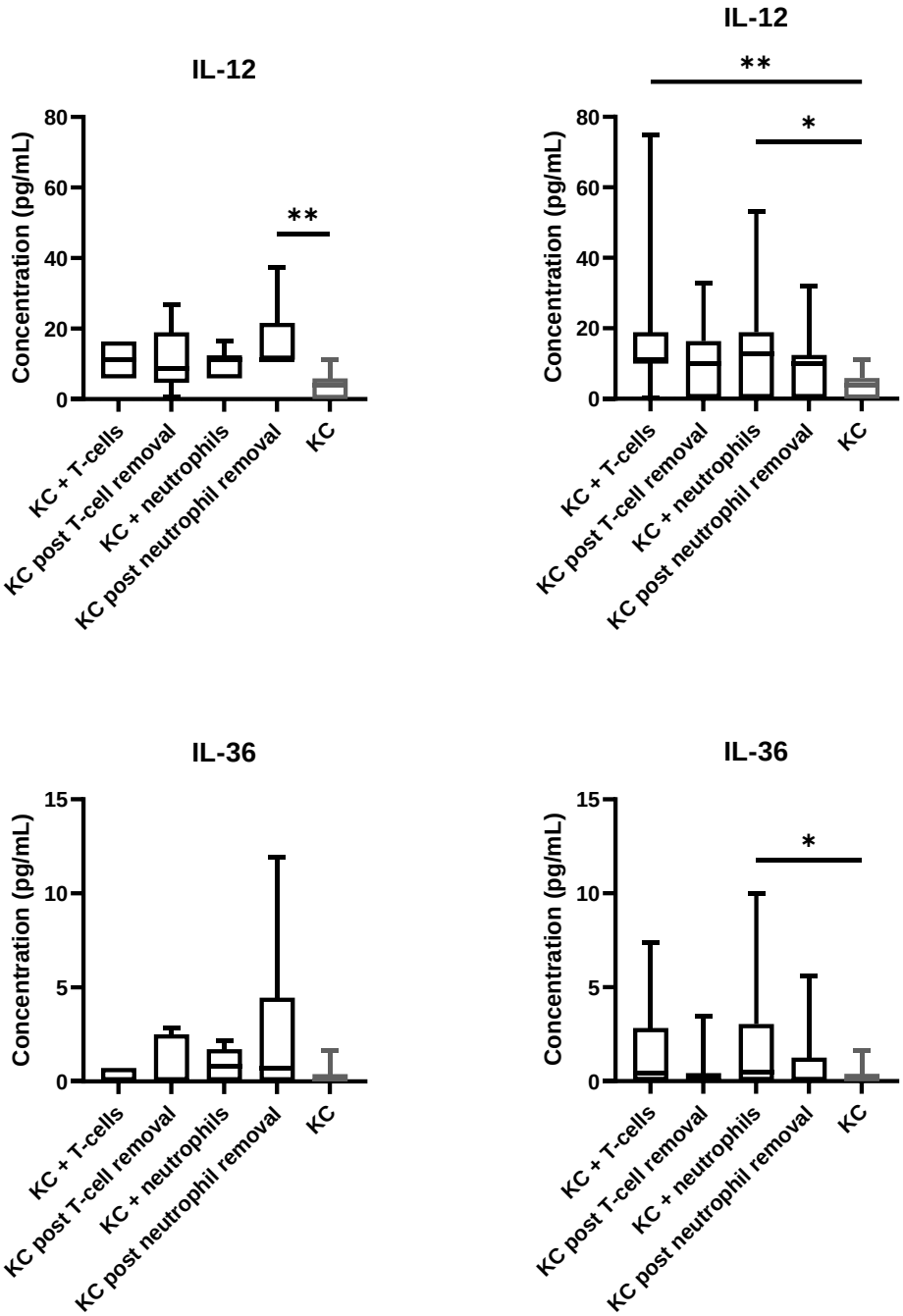


Figure 5.11| Supernatants derived from co-cultures of keratinocytes with psoriatic neutrophils or T-cells were associated with higher levels of pro-inflammatory cytokines and chemokines, when compared to their healthy counterparts.
*A graph showing luminex analysis of CXCL8, CCL2, CCL20, CXCL12, IL-17, TNF, CCL17, CXCL1, CXCL10, IL-12, and IL-36 production levels in supernatants derived from co-cultures of keratinocytes with neutrophils or T-cells derived from either **(A)** healthy controls or **(B)** patients with psoriasis. Prior to supernatant collection, keratinocytes were cultured for 48h (media change and PBS wash at the 24h time point Supernatants from left to right are: 1. KC+T-cells - Supernatant of 24h Keratinocyte and T-cell co-cultures; 2. KC post T-cell removal - Supernatant of 24h Keratinocyte cultures, but prior to this the keratinocytes were co-cultured with T-cells for 24h (KCs from 1); 3. KC+neutrophils - Supernatant of 24h Keratinocyte and neutrophil co-cultures; 4. KC post neutrophil removal - Supernatant of 24h Keratinocyte cultures, but prior to this the keratinocytes were co-cultured with neutrophils for 24h (KCs from 3); 5. Keratinocyte - Supernatant of 24-48h Keratinocyte cultures. Data are presented as box and whiskers plots with the Mean+SD (One-way ANOVA test; *p<0.05; **p<0.01; n.s. = non-significant). n(healthy)=6; n(patients with psoriasis)=29/30; n(keratinocytes only)=8*

5.4.3.2 The role of CXCL8 in neutrophil recruitment by T-cell-conditioned keratinocyte supernatants

Next, we wanted to further address the possible drivers of neutrophil migration, contained within the T-cell-conditioned keratinocyte supernatants. As per Chapter 3, CXCL8 is one of the main drivers of neutrophil migration and likely plays an important role in psoriasis inflammation. Therefore, we wanted to investigate the role of CXCL8 in this setting. To do this, we utilised CXCL8 neutralising antibodies. The antibody concentration needed for CXCL8 neutralisation was firstly optimised in a dose versus response experiment, whereby the response was neutrophil migration induced by 50ng/mL CXCL8. Here, neutrophil transmigration media, containing 50ng/mL CXCL8, was treated with a rising anti-CXCL8 concentration for 30 minutes at 37^0C. The transwell analysis of patient with psoriasis donor-derived neutrophils migration towards 50ng/mL CXCL8, showed that an anti-CXCL8 concentration of over 0.5µg/mL is sufficient to inhibit migration (Figure 5.13). Next, supernatants from CCL20-responsive psoriatic T-cells and keratinocyte co-cultures were treated with 1µg/mL anti-CXCL8. CXCL8 neutralisation was sufficient to reduce the significant increase in neutrophil migration towards 'KC post T-cell removal' supernatants (Figure 5.14). Those supernatants were derived from keratinocytes that were co-cultured with T-cells for a period of 24h; T-cells were then removed and fresh media was given to keratinocytes for further 24h. These data suggest that CXCL8 is key factor, contained within the T-cell conditioned keratinocyte supernatants, in driving the increase in neutrophil transmigration.

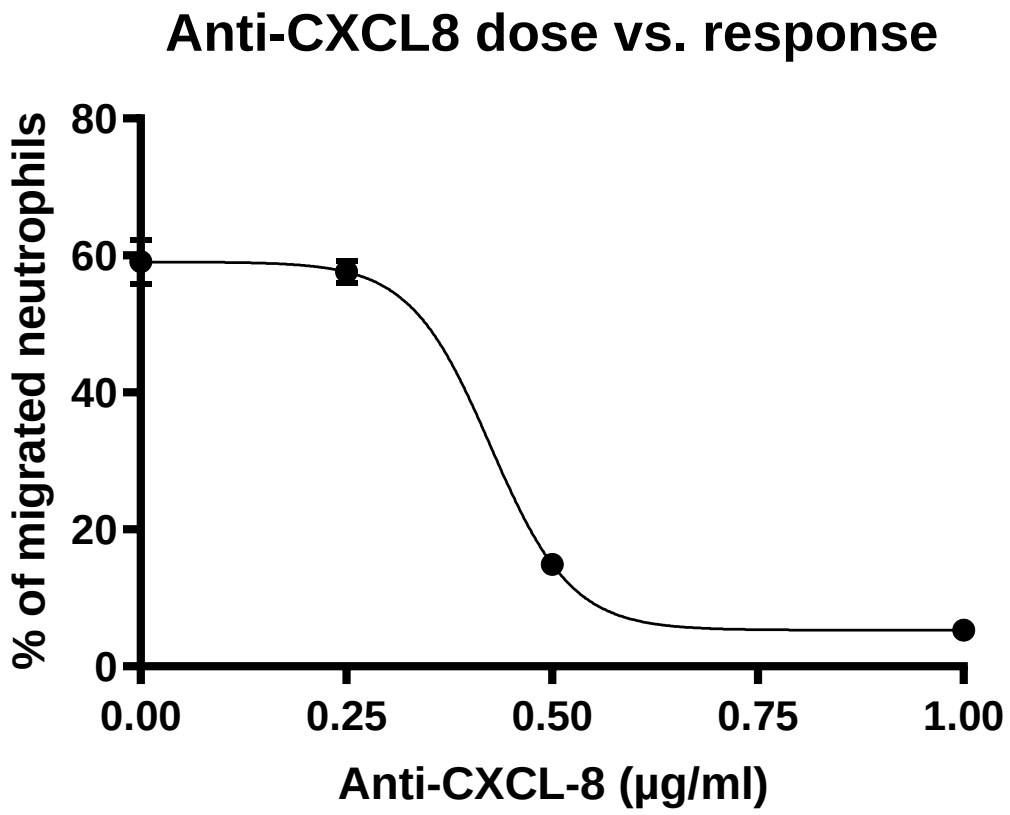


Figure 5.12| Neutralisation of CXCL8 from migration media and subsequent neutrophil transmigration was achieved via the addition of anti-CXCL8 antibody

Dose versus response transwell analysis of required anti-CXCL8 concentration to neutralise 50ng/mL CXCL8 and subsequent neutrophil transmigration. Neutrophils were derived from healthy donors. Prior to neutrophil transmigration, CXCL8 migration media was treated with rising anti-CXCL8 concentration (0-1µg/mL) for 30 minutes at 37°C. Following a 1h incubation period in transwell chambers, migrated neutrophils were collected and counted. Data are presented as inter connected dot plots, with each dot representing the mean of 3 technical replicates, with SD.

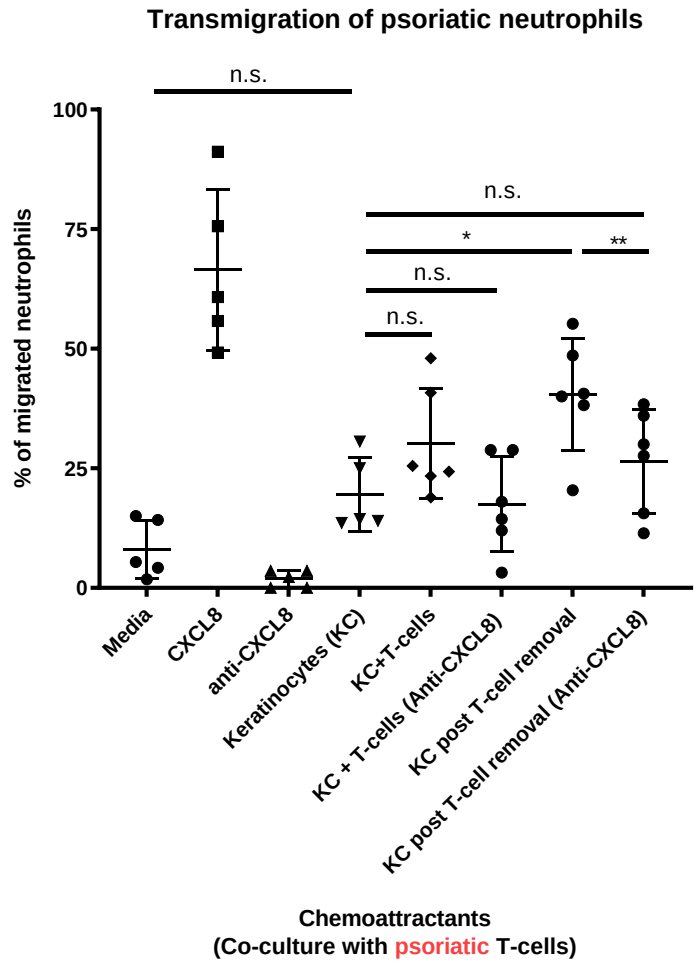


Figure 5.13| CXCL8 neutralisation was sufficient to reduce the significant increase in neutrophil migration towards T-cell-conditioned keratinocyte supernatants

Transwell analysis of the role of CXCL8 in the induction of psoriatic neutrophil migration by T-cell-conditioned keratinocyte supernatant. Psoriatic neutrophils were used to assess the chemoattractive abilities of the T-cell-conditioned keratinocyte supernatants. Prior to transmigration the indicated supernatants were treated with 1µg/mL anti-CXCL8 neutralising antibodies for 30 minutes at 37°C. Following a 1h incubation period in transwell chambers, migrated neutrophils were collected and counted. Keratinocytes for co-culture were derived from at least two different donors. T-cells for co-culture were selected based on CCL20-responsiveness and were derived from 6 different donors, therefore 6 different neutrophil-conditioned supernatants were generated and utilised. The neutrophils of each donor (5 donors in total) were migrated towards 1 to 2 different supernatants. Chemoattractants from left to right are: 1. Media - transmigration media only (negative control); 2. CXCL8 (positive control); 3. Anti-CXCL8 treated media 2; 4. Keratinocyte - Supernatant of 24-48h Keratinocyte cultures; 5. KC-T-cells - Supernatant of 24h Keratinocyte and T-cell co-cultures; 6. Anti-CXCL8-treated supernatant 5; 7. KC post T-cell removal - Supernatant of 24h Keratinocyte cultures, but prior to this the keratinocytes were co-cultured with T-cells for 24h (KC from 5); 8. Anti-CXCL8-treated

*media 7. Data are presented as dot plots, with each dot representing a different biological sample migration, with the Mean+SD (One-way ANOVA test; *p<0.05; n.s. = non-significant)*

5.5 Summary and conclusions

Leukocyte infiltration and maintenance of inflammation in psoriasis is dependent on cellular interactions between leukocytes and leukocytes-stromal cells[11-13]. Importantly, the cellular interactions in the early stages of psoriasis plaque development could pre-determine the formation of pro-inflammatory loops and therefore disease severity[17,707,725]. T-cells and neutrophils are recruited early during plaque development[17,707,725]. However, the sequence of events in terms of chemokine-dependent cell infiltration and leukocyte interactions with stromal cells, remains poorly understood. Understanding the early cellular interactions that lead to leukocyte recruitment to the skin, could help develop more specific treatments for psoriasis.

To better understand leukocyte-stromal cell cross-talk and resultant leukocyte recruitment in psoriasis we set up co-cultures. Leukocytes for co-culture were derived from peripheral blood and selected based on their responsiveness to chemokines. This decision was based on the Chapter 3 finding that psoriatic leukocytes have a heightened migratory potential, and therefore, potentially, a heightened pro-inflammatory phenotype. We also suggest that those leukocytes would be more likely to migrate from peripheral blood to a lesion when *in vivo*. CXCL8-responsive neutrophils and CCL20-responsive T-cells were co-cultured with primary keratinocytes and supernatants were used as chemoattractants. Before co-culturing patient-derived leukocytes with keraticnoytes, we performed a number of optimisation co-culture experiments. For example, we wanted to determine the optimal number of leukocytes for addition to keratinocyte cultures. Due to regulatory reasons, at the time of optimisation, we had to opt for T-cells derived from healthy donors and activate them via antibody stimulation. We demonstrated that exposing keratinocytes to 20,000 activated, but not resting, T-cells for 24 hours causes their detachment and presumably death. This is in keeping with previous studies, which showed that IFNγ, produced by the activated T-cells, was sufficient to induce expression of FasR by keratinocytes[918]. Therefore, the activation induced FasL expression by T-cells may account for the keratinocyte cell death in our co-cultures. To further validate such a hypothesis the expression of the aforementioned molecules needs confirmed and their interactions studies (e.g. by blocking the IFNγ receptor). Interestingly, previous studies have shown that the co-culture

of 50 000 T-cells with keratinocytes does not result in keratinocyte detachment and death[919]. It is important to note, that the optimisation was not repeated using psoriatic T-cells, which is a limitation. Nevertheless, the chosen, after optimisation, number of 5000 leukocytes was well tolerated by keratinocytes, even when leukocytes were derived from patients with psoriasis.

Next, we wanted to determine the phenotype of the CCL20-responsive T-cells to be co-cultured with keratinocytes. We demonstrated that CCL20-responsive T-cells express CD3, CD4, CCR6 and RORγT, which suggests a Th17 phenotype. Importantly, 99.5% of the cells were CD4+ suggesting absence of cytotoxic T-cells and ruling out possible CD8+ T-cell-induced keratinocyte death. Th17 cells are believed to be the main psoriasis driving T-cell phenotype, which orchestrates its action via IL-17/IL-22 production. IL-17 is known to have an effect on keratinocyte function, allowing Th17 cells to participate in the IL-17/IL-23 pro-inflammatory axis[734]. Interestingly, studies have shown that keratinocytes can reinforce Th17 fate when co-cultured with T-cells, which could have implications for skin conditions, such as psoriasis[919]. In agreement with this, we found that CCL20-responsive psoriatic T-cells can condition keratinocytes to produce supernatants that induced a significant increase in the migration of psoriatic neutrophils. This appeared to be a time-dependent process, as supernatants derived from 24h T-cell-Keratinocyte co-cultures did not exert similar effects. Only when T-cells were removed and keratinocytes were given fresh media for further a further 24h period, did this phenomenon occur. This could be explained by the period required for production of neutrophil attracting factors by keratinocyte to happen. Our findings are in keeping with previous co-culture studies that showed CD3/CD28-activated T-cells can induce changes in keratinocyte chemokine production[919]. Importantly, healthy T-cells could not induce similar changes in keratinocyte-driven neutrophil migration via supernatants. This could further suggest that psoriatic T-cells, indeed, have a heightened pro-inflammatory phenotype.

Following this, we wanted to better understand the drivers of neutrophil recruitment contained within the supernatants. The neutralisation of CXCL8 from the T-cell-conditioned media, reduced its ability to induce a significant change in neutrophil migration. This suggests that CXCL8 is an important driver of neutrophil recruitment in psoriasis, which could be affected by the IL-17/IL-23-axis. Interestingly, however, we were not able to detect upregulation of CXCL8, CCL20, and TNF gene expression by T-cell-conditioned keratinocytes at the 48h time point. This could suggest that the expression of these genes is time-dependent and is perhaps not entirely active at the 48h time point (24hours following co-culture). These findings could also suggest for the

existence of differences between mRNA and protein stability. The results could be the consequence of a rapid reduction of mRNA levels, with unchanged protein levels due to high stability of the protein that has been synthesised. Previous studies have reported an increase of chemokine gene expression after 1 day of co-culture with CD8+ T-cells, suggesting that 48h might have been too late[920]. However, differences in the methodology need to be taken into account. For example, the aforementioned study was concerned with CD8+ T-cells and chemokines different to CXCL8 and CCL20. As we discovered that CXCL8 neutralisation from the supernatants diminishes migration, we speculate that the CXCL8 encoding gene was upregulated at an earlier point in time. Nevertheless, the role of other neutrophil-attracting molecules, such as CXCL1 and neutrophil-activating peptide-2, remains to be examined.

The supernatants we used for these experiments were also outsourced for Luminex analysis of CXCL8, CCL2, CCL20, CXCL12, IL-17, TNF, CCL17, CXCL1, CXCL10, IL-12, and IL-36 production levels. The findings from the Luminex analysis suggested that the peak of pro-inflammatory chemokine production (e.g. CXCL8) in fact takes place during the first incubation period of 24h, when both leukocytes and keratinocytes are present in the cultures. This would suggest that the primary producers of these chemokines are the leukocytes, rather than the conditioned keratinocytes. Furthermore, chemokine levels return to close to baseline levels at the 48h timepoint, again supporting the hyopothesis above. However, as discussed earlier, the sequential transmigration experiments showed that it was in fact the 48h time point supernatants that were more potent at inducing leukocyte migration. It is important to mention that over-stimulation with chemokines can inhibit leukocyte migration, which could explain why lower migration levels were observed at the 24h time period. The supernatants, however, contain many other pro-inflammatory cytokines and chemokines, which could potentially act on the same receptors and lead to over-saturation and thereby a loss of directed migration required to move into the upper transwell chamber. Nevertheless, these data clearly demonstrate that psoriatic T-cells and neutrophils are more potent producers of pro-inflammatory proteins, than their healthy counterparts.

We also found that CD4+ T-cells which were not CCL20-selected, could not condition keratinocytes to produce supernatants that were chemoattractive to neutrophils. This suggests that T-cells would need to be of a particular CCL20-responsive phenotype in order to be able to induce such changes in keratinocyte production profile. A possible explanation for this could be the presence of regulatory T-cells (Treg), which could supress keratinocyte production of neutrophil attracting factors. CCL20-selection could limit Treg presence and therefore allow

stronger keratinocyte stimulation. Alternatively, this could be explained by the relative absence of CCL20-responsive cells, which are in the minority of cells present when looking at a whole CD4+ T-cell population.

However, regardless of this, previous research has shown that regulatory T-cells do not have an effect on chemokine production by keratinocytes[919]. Nevertheless, our data showed that not all of the CCL20-responsive T-cells were ROR-γt positive, suggesting the presence of a different CCR6+ T-cell phenotype. Interestingly, previous research has reported that an immunosuppressive microenvironment, such as that in cancer, favours the maintenance and recruitment of CCR6+ Treg cells[921]. This, however, would not be a likely possibility in psoriasis as Treg cells have been shown to have a strongly subdued activity. This can be explained by the presence of pro-inflammatory loops, which favour the predominant production of factors which stimulate the development of Th17 phenotype.

We then explored the possibility of a neutrophil-driven keratinocyte-dependent auto-amplification of neutrophil recruitment. We demonstrated that keratinocytes conditioned by CXCL8-responsive psoriatic neutrophils could not induce a significant change in the migration of psoriatic neutrophils. This suggests that neutrophils are not able to initiate a keratinocyte-dependent, neutrophil-recruitment auto-amplification axis on their own. Importantly, neutrophils could be directly involved in orchestrating self-autoamplification by producing CXCL8 themselves, as demonstrated by other studies[922]. Interestingly, CXCL8-responsive neutrophils (psoriatic and healthy co-cultured with keratinocytes could not induce the production of supernatants able to drive significant changes in T-cell (psoriatic and healthy) migration. This is in contrast to previous studies, which have demonstrated that neutrophils at the lesions can release keratinocyte-stimulating factors, such as IL-17[825]. It has been previously hypothesised that this could be a possible route for the induction of chemokine production and T-cell recruitment by keratinocytes. Nevertheless, our experiments were carried out in an *ex vivo* setting and cannot therefore fully replicate the psoriasis lesion microenvironment in humans. Based on our model, however, we propose that the early events in psoriasis plaque development are more likely to dependent on T-cell-orchestrated neutrophil recruitment rather than vice versa. This could possibly be attributed to memory T-cells with a resident skin status that have a Th17 phenotype.

Chapter 6: Discussion

6.1 Introduction

The contribution of the immune system to the pathology of psoriasis has been the main focus of research in the field ever since the successful therapeutic use of immunosuppression demonstrated that psoriasis is immune-mediated. It is now clear that leukocytes and their effector molecules are critical to the development of a psoriatic plaque[2,13,726]. The majority of the approved agents for the treatment of psoriasis target immune cell function non-specifically, or target selected pro-inflammatory cytokines (e.g. TNF, IL-23, IL-17). Although these have proven effective, new evidence suggests that the targeting of different immune pathways, such as immune cell migration, might improve treatment specificity[798,853]. Work has highlighted the importance of chemokines, drivers of leukocyte migration, in psoriasis pathogenesis and their role as modulators of severity[853]. Leukocyte migration is a key early event in psoriasis, and it has become clear that leucocyte positioning within the skin is important for the development of the psoriatic plaque[798]. Additionally, some of the most efficacious biologic agents available appear to profoundly impact leukocyte positioning in skin, though this finding appears coincidental rather than the intention of the therapy[725]. However, an effective leucocyte chemotaxis targeting agent is yet to be developed. In addition, once the self-amplifying pathogenic loop is set in motion in the skin, psoriatic plaques do not resolve without treatment, whilst resident tissue memory cells increase the likelihood of plaques recurring in the same site. Targeting leukocyte migration, and thus the prevention of the plaque forming in the first place, could be a potential means by which the disease course of psoriasis can be altered.

The overall aim of this thesis was to investigate the chemokine-dependent orchestration of the immune response in psoriasis. This study benefited from the unique access to one of the largest biologic psoriasis cohorts in the world (in Leeds) and the ability to explore a multitude of different drugs, using real-world samples. We wanted to develop an *ex vivo* psoriasis-resembling model for studying key aspects of leukocyte migration in a systematic and reductionist manner. This was an important first step as the assay would allow us to address specific questions regarding leukocyte migration. For example, we wanted to investigate the migratory potential of psoriatic leukocytes and the chemokine/receptor couple(s) which orchestrate leukocyte recruitment to the lesions, as despite chemokines having been previously found in psoriatic skin, little is known about their direct effect on leukocytes in psoriasis. Furthermore, we wanted to understand the effect of approved drugs for the treatment of psoriasis on leukocyte migration. Finally, this study sought to elucidate the sequence of migratory

event which take place during early plaque development. It is important for such questions to be answered, as new therapeutic targets need to be identified. Importantly, the chemokine/ chemokine receptor network is dynamic and a different chemokine or set of chemokines might drive pathogenesis in different people. Although this is just one example, many factors need to be considered prior to targeting specific pathways for the treatment of psoriasis.

The aims of this thesis were to:

5. Develop an assay which would allow for the selection of leukocytes, more likely to migrate to a psoriatic lesion, from peripheral blood
6. Investigate the migratory potential of leukocytes and ability of psoriasis-associated chemokines to attract leukocytes in the context of psoriasis
7. Investigate the effects of different exemplar anti-psoriatic drugs for the treatment of psoriasis on leukocyte migration
8. Investigate chemokine-orchestrated leukocyte-stromal cell interactions in psoriasis

6.2 Neutrophils and T-cells derived from patients with psoriasis have an enhanced migratory potential

In this chapter, we sought to evaluate the migratory potential of leukocytes and chemokine function in psoriasis. It has been previously shown that the production of multiple chemokines is highly elevated in the psoriatic lesion. However, few studies have addressed the poise of leukocytes to migrate and the direct effect of psoriasis-associated chemokines on leukocyte migration. The effects of current drug treatments on these events is also unclear. The main aims of this chapter were to:

- Develop an *ex vivo* psoriasis-resembling assay for studying leukocyte migration
- Use the assay to study leukocyte migration and identify chemokines apparently key to the recruitment of leukocytes to psoriasis lesions
- Use the assay to define the differences between the migratory potential of healthy donor-derived and patients with psoriasis-derived leukocytes

- Use the assay to study the effect of different key exemplar drugs for the treatment of psoriasis on leukocyte migration

In this chapter, we **hypothesised that chemokines, expressed highly in psoriatic lesions, have the ability to attract neutrophils and T-cells from the peripheral blood of patients with psoriasis.** Importantly, the increase in neutrophil numbers in the plaques could be explained by a concomitant increase of neutrophil chemotactic factors and neutrophil peripheral blood levels[897]. However, the systemic increase of pro-inflammatory cytokines in psoriasis could potentiate leukocyte recruitment to the lesions by affecting leukocyte migratory potential. Therefore, it was further **hypothesised that the increase of neutrophil numbers in the plaque is due to the cells being primed for migration in the periphery, rather than reflecting their increase in blood.**

In order to study these hypotheses, transwell permeable supports (TPS) were utilised. To achieve a more psoriasis-like setting, we selected and optimised psoriasis-associated chemokines and isolated leukocytes from the peripheral blood of patients with psoriasis. Neutrophils are known to be highly responsive to CXCL8[898]; however, they have been shown to upregulate various chemokine receptors, such as CCR2 and CXCR6, in the context of inflammation[837,899]. This suggests that their recruitment to the psoriatic lesion may rely on multiple chemokine receptors; however, this has received limited attention in the field. We showed that CCL2 and CXCL16 do not attract neutrophils derived from patients with psoriasis or healthy donors, but CXCL8 does. CXCL8 induced the highest rate of neutrophil migration and also had an effect on neutrophils derived from healthy donors. However, neutrophils derived from patients with psoriasis had an enhanced responsiveness to CXCL8, when compared to neutrophils derived from healthy donors. This suggests that the increase of leukocytes in the plaques is not just due to an increase in chemokine levels and peripheral leukocyte numbers, but due to changes in migratory potential. We propose that this is an important finding, as it may suggest that increased leukocyte migratory potential is a mark of psoriasis-associated systemic inflammation. Early studies demonstrated that neutrophil chemotactic activity could be enhanced by preincubation with human plasma from patients with extensive psoriasis lesions; however, this was not possible when the plasma was derived from healthy donors[896]. These data are in support of our discoveries and hypotheses and suggest that neutrophils in psoriasis might experience an increase in sensitivity to CXCL8. Irrespective of our findings, it should be noted that neutrophils are fast responders and are quick to up-regulate pro-inflammatory receptor expression and cytokine production[300,361,396]. Therefore, albeit our best efforts of careful handling and isolation of neutrophils, their activation state could have been affected. Nevertheless, both neutrophils

derived from healthy and patients with psoriasis-donors were handled in the same manner, yet there were significant differences between the groups. Our findings of neutrophil response to CXCL16 are not in keeping with other studies. It has been shown that 1000ng/ml of CXCL16 can mediate chemotaxis of neutrophils; however, we deem this to be such a high concentration so as to be of questionable relevance physiologically[837]. Interestingly, CXCL16 was shown to play an important role in enhancing CXCL8-induced neutrophil migration, by the same group. This suggests that chemokines in the context of psoriasis might act co-operatively to induce the inflammatory response. Nevertheless, it must be noted that the response of neutrophils to chemokines *in vivo*, where a myriad of other factors are at play, might differ. The *in vivo* ability of neutrophils to respond to different chemokines might provide them with the means to infiltrate different skin compartments (e.g. epidermis, dermis). Although CXCL8 is the main chemokine known for neutrophil recruitment, the role of other psoriasis-associated chemokines needs to be further elucidated.

Similarly to neutrophils, T-cells up-regulate the expression of specific chemokine receptors during inflammation, but the migratory mechanism in the context of psoriasis is unclear[2,760,830]. Key studies have reported the increased production of multiple potential T-cell attracting chemokines in the psoriatic lesion (Table 2 in the introduction), though their functional role is unclear[2,760,830,902]. We speculate that the high number of such possible chemokines reflects the heterogeneous population of T-cells present at the lesion (e.g. a specific set of chemokines drives CD8+ T-cell accumulation in the epidermis). A limitation to this study was the initial (during optimisation) reduced ability to further recruit patients with psoriasis. During this time period, we diversified our experimental methodologies and e.g. used resting and activated healthy T-cells in the transwell experiments, which would later serve as negative and positive controls, respectively. Our data suggest that resting healthy T-cells have a limited migratory capacity and thus fail to migrate towards most of the psoriasis-associated chemokines. We suggest that the phenotype of T-cells derived from patients with psoriasis will be closer to this of activated healthy T-cells. Indeed, we demonstrate that activated healthy T-cells have higher migratory capacity than their resting counterparts. The ability of CCL5, CXCL10 and CCL20 to attract T-cells derived from patients with psoriasis was later confirmed. The observation that CCL5 can induce migration of patients with psoriasis-derived, but not healthy donor-derived activated, T-cells is important. This finding demonstrated that the *ex vivo* activation of healthy T-cells cannot fully replicate the phenotype of T-cells derived from patients. Furthermore, T-cells derived from patients with psoriasis have an enhanced responsiveness to CCL20 (200ng/mL), when compared to T-cells derived from healthy donors. This finding is in agreement with the increase in neutrophil (from patients with psoriasis) migratory responsiveness to CXCL8. Further work is necessary to

explore the roles of chemokines other than CCL5, CXCL10, CCL20, in the context of psoriasis pathogenesis.

We then explored the migratory potential of leukocytes derived from patients with diseases that share key features with psoriasis, such as neutrophilic dermatoses (NDs) and psoriatic arthritis (PsA). Our data suggest that neutrophils, but not T-cells, derived from patients with NDs had an increase in their responsiveness to CXCL8 and CCL20, respectively. Our data further highlights the similarities in pathogenesis that psoriasis and NDs share. This is important, as it might help inform the development of future therapies for the treatment of NDs. Nevertheless, it is important to note that we carried out our studies on a limited number of ND patients, which are inherently a heterogenous cohort (e.g. these conditions are typically rare, and immunocompetent patients on no immunomodulators are exceedingly rare). Therefore, this is a limitation which prevents us from drawing generalisable conclusions. A future remedy would be the recruitment of more patients, enabling more sophisticated patient stratification and sub-group analyses. Furthermore, our data showed that neutrophils derived from patients with PsA have an increased responsiveness to CXCL8, which is in support of previous findings[923]. Interestingly, and in contrast to previous reports, T-cells derived from patients with PsA did not exhibit an increase in migration in our studies. This could suggest that CCL20 acts in conjugation with other chemokines to induce T-cell recruitment in the context of PsA. Nevertheless, it has been suggested that much like neutrophils, the T-cells from patients with PsA are more migratory than their healthy counterparts[923]. We also discovered that the migratory potential of leukocytes is not related to the PASI score (measuring objective disease severity) of patients with psoriasis, nor is their age or sex. However, we speculate that these relationships could change if more patients are recruited in the future. Finally, we examined the effect of different in vivo psoriasis treatment strategies on leukocyte migration. Treatment of patients with psoriasis with Apremilast reduces the ability of neutrophils to respond to CXCL8, but responsiveness of T-cells to CCL20 is unaffected. Apremilast is a PDE4 inhibitor, which aids the suppression of multiple pro-inflammatory cytokines and provides a systemic relief of inflammation. We explore the mechanisms behind this key observation in the next chapter.

Main conclusions from chapter 3:

- We have established an *ex vivo* transmigration system to study leukocyte migration in the context of psoriasis.

- Not all chemokines are key to leukocyte plaque-homing, but CXCL8 and CCL20 plays an important role
- Psoriasis-derived leukocytes exhibit a unique enhanced migratory capacity.
- There are apparent differences in T-cell responsiveness to CCL20 when comparing plaque psoriasis and PsA
- Apremilast treatment of patients with psoriasis reduces neutrophil migratory potential

6.3 Apremilast diminishes neutrophil migration in response to CXCL8 stimulation

Based on the finding that the treatment of psoriasis patients with Apremilast leads to the reduction of neutrophil migratory capabilities; we decided to further investigate the effect of Apremilast on leukocyte migration and define a possible molecular mechanism of action. Understanding the effects of Apremilast on leukocytes, important to the inflammatory process in psoriasis, could be a useful tool for predicting a patient's responsiveness to the drug. Determining if a drug would be efficacious in treating a patient could be critical for achieving optimal treatment results. The main aims of this chapter were to:

- Determine the effect of Apremilast on neutrophil responsiveness to a key psoriasis-related chemokine - CXCL8
- Investigate Apremilast's mode of action in restricting neutrophil migration
- Investigate the effect of Apremilast on leukocyte migration in the Imiquimod murine model of psoriasis

Apremilast acts by inhibiting phosphodiesterase 4 (PDE 4) activity, which is an enzyme that breaks down cyclic adenosine monophosphate (cAMP)[126]. The increase in cAMP activity leads to the downregulation of pro-inflammatory mediators' expression, which in turn alleviates disease symptoms[124-126]. Therefore, Apremilast can exert broad anti-inflammatory effects, as pro-inflammatory cytokines are major drivers of inflammation. This suggests that it is possible for Apremilast to subdue neutrophil migratory potential, and therefore plaque infiltration, indirectly. Based on this, **it was hypothesised that Apremilast restricts neutrophil influx into psoriasiform skin; reduces expression of key pro-inflammatory mediators and psoriasiform inflammation**. However, studies have shown that PDE4 inhibition in leukocyte can abrogate migration and abolish important to migration intracellular pathways, such as calcium fluxes[664,685,687-689]. Therefore, Apremilast could also have a direct effect on neutrophils and their ability to migrate, by interfering with intracellular signalling pathways. **It was**

hypothesised that *ex vivo* treatment of neutrophils with Apremilast, reduces CXCL8 responsiveness. We further hypothesised that Apremilast abolishes intracellular calcium flux in response to CXCL8 by acting on the Ca^{2+} intracellular pathway.

In order to investigate the effect of systemic Apremilast administration on neutrophil influx into plaques and psoriasiform inflammation, we utilised the imiquimod mouse model of psoriasis. Here we show that treatment with Apremilast can significantly reduce the influx of neutrophils ($Ly6G^{hi}Ly6C^{int}$ cells) into psoriasiform inflamed skin of C56BL/6 mice. Furthermore, Apremilast-treated mice had a lower modified PASI score and epidermal thickness than Apremilast-untreated mice with psoriasiform inflammation and were rescued from a significant weight loss. These data suggest that Apremilast-induced alleviation of psoriasiform inflammation could be, in part, related to reduced neutrophilic skin influx. However, this experiment was carried out once, albeit on 5 mice in each group, and reproducibility needs addressed (e.g. by repeating it). It is important to mention that this model of psoriasiform inflammation has its limitations, especially when studying skin thickness, and though it recapitulates key aspects of psoriasis, it does not replicate all aspects. This is because artificial increase or decrease of acanthosis may occur when sectioning the skin. Furthermore, the modified PASI score can also be a subject of bias. Nevertheless, it is one of the most utilised models of psoriasiform mouse inflammation that we have available for research purposes. Multiple previous studies have reported for its accuracy and reliability[210,721,754]. It is also important to note that this experimental approach is not a strict representation of Apremilast treatment of patients with psoriasis. For example, we were administering Apremilast and applying imiquimod at similar time frames, which rarely happens in humans (treatment is usually commenced well after plaque development, sometimes decades later). Although Apremilast is not given to patients prior to lesion development, this could highlight the benefits of having an early treatment strategy. Furthermore, Apremilast was hard to dissolve in the carrier and a minimal portion of it would precipitate. This is important as it might have prevented the mice from fully absorbing the drug. Although such challenges might seem minor, appropriate data interpretation requires their careful consideration. The dosing and gavage of the drug were carried out in keeping with previous studies[917].

We also investigated the direct effect of Apremilast treatment on neutrophil responsiveness to CXCL8 by utilising the migration assay we developed earlier. Our data showed that Apremilast can directly inhibit the responsiveness of neutrophils, derived from patients with psoriasis, to CXCL8. Our findings are in agreement with previous reports that some PDE4 inhibitors can affect neutrophil function and migration directly[664]. However, these reports were not related to neutrophils derived from patients with psoriasis, their response to CXCL8 and did not include

Apremilast but other compound of little or no clinical significance. Nevertheless, the common outcome of PDE4 inhibition must be noted. Furthermore, some of the experiments were carried out in the context of an inflammatory condition, albeit not psoriasis, which allows for this comparison to be drawn[664]. The expression of CXCR2 (a CXCL8 receptor) on neutrophils remained unchanged for at least two hours post treatment. Although this would suggest that Apremilast does not abrogate neutrophil migration via CXCL8 receptor inhibition, certain aspects of this need to be considered. For example, changes in chemokine receptor expression take longer than 2 hours and could therefore be affected by a longer treatment period[914]. This, however, would be difficult to achieve as neutrophils have a very short lifespan, especially *ex vivo*, and are rapid to change their gene expression signature[924].

This prompted us to investigate other possible mechanisms. For example, PDE4 inhibition has been previously linked to disturbances of the calcium flux system in leukocytes[687]. Importantly, other studies have linked the absence of calcium fluxes to abolished neutrophil migration. Here we demonstrate that Apremilast can inhibit neutrophil intracellular Ca^{2+} transients in response to CXCL8. However, it remains unknown how the intracellular Ca^{2+} transients in response to CXCL8 may be lost and how calcium removal causes inhibition of migration. Studies have tried to characterise possible pathways. For example, the induction of up-regulated endomembrane Ca^{2+}-ATPase activity via cAMP-dependent PKA has been proposed[685,687-689]. Although this is one possible mechanism, PKA has been associated with the activation of multiple other molecules important for leukocyte migration[654,664,687]. Furthermore, studies regarding the role of cAMP-induced PKA in leukocyte migration are contradictive, as both migration inhibitory and activation properties have been attributed to the pathway[652-656]. What is even more striking is that cAMP-mediated modulation of neutrophil migration might actually rely on another cAMP effector protein – Epac-1[658,659,664]. Alternatively, cAMP-dependent PKA activation, as a result of Apremilast treatment, might act to abolish calcium transients needed for the induction of migration. Perhaps, deciphering the sequence of events of this pathway would provide a sensible explanation. It is important to mention that we used the non-ratiometric dye Fluo-4, when detecting intracellular Ca^{2+} fluxes. Non-ratiomteric dyes show a large increase in fluorescence intensity upon Ca^{2+} binding, indicative of and directly related to an increase in Ca^{2+} concentration. However, when using non-ratiometric dyes, factors not related to Ca^{2+}, such as acquisition conditions, probe concentration and optical path length can influence the intensity of the signal. For this reason, our data is qualitative, rather than quantitative. Using ratiometric dyes as calcium indicators could be considered as means to achieve data quantification.

Main conclusions from chapter 4:

- Apremilast can directly affect neutrophil responsiveness to CXCL8
- The loss of CXCL8 responsiveness could be linked to abrupt intracellular Ca^{2+} regulation, as Ca^{2+} fluxes in response to CXCL8 were also lost
- Apremilast treatment can alleviate psoriasiform inflammation *in vivo* and this could be related to a reduction of neutrophil influx into psoriasiform skin

6.4 Sequential order of neutrophil-keratinocyte-T-cell interactions in psoriasis pathogenesis

In the previous chapters, we demonstrated and highlighted the importance of neutrophils and neutrophil and T-cell migration to the inflammatory process in psoriasis. However, neutrophils and other leukocytes, such as T-cells, are a part of a big network of cells and interactions that determine the course of disease development[11-13]. In this chapter, we sought to investigate the outcome of key stromal-cell/leukocyte interactions in psoriasis. Although immune cell-induced keratinocyte changes have been previously studied, the early events of psoriasis plaque development are unclear[17,707,725]. The sequence of events, in particular, has been and still is a poorly understood matter. Therefore, the main aims of this chapter were to:

- Establish stromal-leukocyte co-cultures
- Determine if T-cell-conditioned keratinocytes are responsible for the recruitment neutrophils or if neutrophil-conditioned keratinocytes are responsible for the recruitment T-cells
- Define the differences between the KC secretome, induced by T cells and neutrophils

A pivotal interaction in psoriasis pathogenesis, which is likely largely chemokine-dependent, is between T cells, neutrophils and keratinocytes[72,568,825,827]. Recent findings have identified T helper 17 (Th17) cells and the IL-23/IL-17 axis as central to the development of psoriasis pathology[721,762]. This inflammatory axis is particularly important, as it stimulates keratinocytes to produce neutrophil and Th17-attracting factors, such as CXCL8 and CCL20, respectively[765,766]. Considering that Th17 cells could have a resident status in the skin, this could potentially be an early mechanism of leukocyte recruitment to the plaques[699,785]. Therefore, it was hypothesised that **skin-targeting CCL20-responsive T-cells, from patients with psoriasis, interact with keratinocytes to orchestrate subsequent neutrophil and T-cell recruitment in a chemokine-dependent manner.** Further to this, neutrophils also arrive early at the lesions and

store and produce a plethora of pro-inflammatory factors[25,722]. For example, neutrophils produce CXCL8 and store IL-17 (feeding into the IL-17/IL-23 axis), which can lead to the recruitment of leukocytes[723,825]. This led us to hypothesise that **skin targeting CXCL8-responsive neutrophils, from patients with psoriasis, interact with keratinocytes to orchestrate subsequent neutrophil and T-cell recruitment in a chemokine-dependent manner.** Deciphering these cellular interactions will give us a better insight into disease initiation and progression, potentially unmasking novel strategies for future therapy development.

To test these hypotheses, we established leukocyte-keratinocyte co-cultures and utilised the migration assay we developed. Neutrophils and T-cells were derived from patients with psoriasis and were selected from peripheral blood based on responsiveness to CXCL8 and CCL20, respectively. Further to this, we reasoned that chemokine responsive leukocytes, would be more likely to be recruited to the plaques of patients with psoriasis. Primary keratinocytes were used in these co-cultures. Herein, we demonstrate that exposing keratinocytes to 20,000 activated, but not resting, T-cells for 24 hours causes their death. This is in keeping with previous studies, which showed that IFNγ produced by the activated T cells, was sufficient to induce expression of FasR by keratinocytes[918]. Therefore, the activation induced FasL expression by T cells may account for the keratinocyte cell death in our co-cultures. To further validate such a hypothesis the expression of the aforementioned molecules needs confirmed and their interactions studies (e.g. by blocking the IFNγ receptor). An obvious limitation of such co-cultures would be the possibility of a CD8+ T-cell-mediated keratinocyte killing. However, it is important to mention that CD8+ T-cell responses require the presence of local signals, such as IFN-γ, for the activation of their cytotoxic functions[585,586,925]. Such signals are most commonly provided by dendritic cells, which were also absent from the co-cultures[585,586]. Furthermore, the CD8+ T-cell would also need to be specific for the antigens expressed by the keratinocytes, which would be a highly unlikely event in this case. Nevertheless, contact free co-cultures could be utilised to negate contact dependent killing. In such an instance, however, it would be important to consider the potency of the T-cell stimulatory signals to keratinocytes. It was decided that 5000 leukocytes would be used in keratinocyte co-cultures, as they did not induce cell death. The generated *in vitro* co-culture supernatants were then used as chemoattractants in our novel sequential migration assay. For example, supernatants from neutrophil-conditioned keratinocytes were used as T-cell chemoattractants and vice versa.

Our data suggested that CCL20-responsive CD3+CD4+CCR6+RORγT+ T-cell, derived from patients with psoriasis, can condition keratinocytes to produce supernatants which induce a significant increase in the migration of neutrophils, derived from patients with psoriasis. This was

a time-dependent phenomenon, which could be explained by the period required for production of neutrophil attracting factors by keratinocyte to happen. Previous research has also shown that keratinocytes can produce multiple chemokines as a result of co-culture with T-cells[919]. This is important, as it demonstrated the cross-talk between leukocytes and stromal cells in the skin. The ability of T-cells to induce such an effect was restricted to patient with psoriasis-derived T-cells. Furthermore, CD4+ T-cells, derived from patients with psoriasis, which were not CCL20-selected could also not lead to the generation of neutrophil attracting keratinocyte co-culture media. These findings are also very important. Once more the heightened pro-inflammatory state of patients with psoriasis-derived leukocytes and requirement for a specific T-cell phenotype for psoriasis pathogenesis was demonstrated. It could be argued that by omitting CCL20 selection, we have introduced regulatory T-cells to the co-cultures. However, regulatory T-cells in psoriasis have been shown to have a strongly subdued activity[794,919]. Nevertheless, regulatory T-cells have been shown to express CCR6 in certain scenarios (e.g. cancer), so this could be a possibility to be explored[921]. It would be interesting to investigate the FOXP3 expression profile of the CCL20-selected T-cells we used for co-cultures; however, as mentioned psoriasis regulatory T-cells have limited effector functions. What is more striking is that there is tendency for regulatory T-cells in psoriasis to assume a Th17 phenotype[794]. To better investigate the effect of each T-cell phenotype on keratinocyte function, flow-cytometry cell sorting could be utilised prior to co-culture establishment. Critically, the neutralisation of CXCL8 from the supernatants alleviated their ability to induce a significant change in neutrophil migration. This evidence serves to demonstrate the critically important role of CXCL8 in psoriasis pathogenesis and neutrophil recruitment. This finding is in agreement with previous studies, which have demonstrated the relevance of CXCL8-induced neutrophil recruitment in the context of psoriasis[898,907].

Contrary to these findings, CXCL8-responsive neutrophils co-cultured with keratinocytes could not induce the production of supernatants able to drive significant changes in T-cell or neutrophil migration. This suggest that a CXCL8-dependent neutrophil auto-amplification loop in psoriasis might not exist; or if it does it might depend on the co-operation of CXCL8 with other chemokines. Nevertheless, our experiments were carried out in an *ex vivo* setting and cannot therefore fully replicate the psoriasis microenvironment. Based on these findings, we propose that the early events of psoriasis plaque development are more likely to dependent on T-cell-orchestrated neutrophil recruitment rather than vice versa. This could possibly be attributed to memory T-cells with a resident skin status that have a Th17 phenotype, as these cells are easy to activate and potent cytokine producers.

Main conclusions from chapter 5:

- CCL20-responsive T-cells, derived from patients with psoriasis, can condition keratinocytes to produce supernatants which induce a significant increase in the migration of neutrophils, derived from patients with psoriasis
- This is a time-dependent process
- T-cells derived from healthy donors could not induce similar changes in keratinocyte-driven neutrophil migration via supernatants.
- The neutralisation of CXCL8 from the T-cell-conditioned media, reduce its ability to induce a significant change in neutrophil migration.
- CD4+ T-cells not selected on CCL20 migration could not condition keratinocytes to produce supernatants, which induce significant changes in neutrophil migration.
- Keratinocytes conditioned by CXCL8-responsive neutrophils, derived from patients with psoriasis, could not induce a significant change in the migration of neutrophils or T-cells, derived from patients with psoriasis

6.5 Conclusions and future directions

During the course of this study, we have developed an assay which has allowed the interrogation of leukocyte migration in the context of inflammatory conditions, such as psoriasis. We demonstrated that CXCL8 and CCL20, CCL5, CXCL10, play an important role in driving neutrophil and T-cell recruitment, respectively, to the psoriasis lesions. We found that this was not the case for other chemokines, such as CXCL16 and CCL2, but we suggest their effects might become apparent when acting in conjugation with other chemokines. We have shown that neutrophils and T-cells, derived from patients with psoriasis, have an increased responsiveness to psoriasis-associated chemokines CXCL8 and CCL20, respectively. We also demonstrated that this phenomenon is present in psoriatic arthritis neutrophils, but not T-cells and can be affected by drug treatments.

Further work is required to fully elucidate the potential clinical relevance of these findings and establish migratory potential as a possible marker of inflammation in psoriasis. For example, finding a link between the increase in migratory potential and patients' psoriasis area severity index could be an important step; however, that would require the recruitment of a large number

of participants. An important future objective would be to investigate the co-operative action of different chemokines in the recruitment of leukocytes in the context of psoriasis. Further to this, it is important to consider the effect of the local microenvironment on chemokine receptor expression. We speculate that upon arrival at the psoriatic lesions, the leukocytes could upregulate or downregulate the expression of certain chemokine receptors. Therefore, even if a particular chemokine receptor is important for homing to the dermis, a different set of receptors could guide the cells into the epidermis. Defining the role of factors which may trigger such changes is therefore an important future objective. Investigating the role of psoriasis-related chemokines and their adjacent receptors in leukocyte recruitment to the epidermis is critical for the identification of new therapeutic targets.

In this thesis, we have demonstrated that Apremilast treatment of patients with psoriasis diminishes the migratory potential and CXCL8-responsiveness of their neutrophils. This is an important finding, as it might tell us why some patients respond better to the drug than others, shall this be linked to leukocyte migration. We have also confirmed that Apremilast directly affects neutrophil migration in an ex vivo setting. The findings in this thesis suggest that Apremilast exerts these effects by acting on the intracellular calcium flux system. We also provide evidence that Apremilast alleviates inflammation and neutrophil skin influx in the imiquimod murine model of psoriasiform inflammation. These findings strongly suggest that the success of in-use medications for the treatment of psoriasis could be related to the attenuation of leukocyte migration.

An important future objective is to unravel the intracellular signalling pathways, affected by Apremilast, resulting in attenuation of migration. We have made an attempt on addressing the effects of Apremilast on the PKA system but due to the COVID-19 crisis this could not be interrogated in detail within the timeframe of the project as had been hoped. Therefore, further work is required to optimise and carry out that experiment successfully. Interrogating the intracellular signalling pathways that govern leukocyte migration in response to Apremilast is important, as they might differ between patients and therefore impact treatment efficacy. Furthermore, the effects of other drugs, such as Methotrexate, on leukocyte migration should be further investigated in the context of autoimmunity. It would be beneficial for the studies in the context of murine inflammation to be repeated, as reproducibility needs addressed. In addition to this, the gene expression studies on the mouse skin samples remain highly inconclusive and should be addressed. It would furthermore be beneficial to collect mouse skin samples, at a different time point.

We have also demonstrated, via the utilisation of co-cultures, that CCL20-responsive T-cells are able to induce the production of neutrophil-attracting factors by keratinocytes. These co-cultures were set up after multiple optimisation steps and allowed us to study the sequential migration of leukocytes. We have also shown that CXCL8 is one of the pro-inflammatory factors, contained within the T-cell-conditioned keratinocyte media, driving neutrophil recruitment. Our data also showed that neutrophil-conditioned keratinocyte media was unable to induce neutrophil and T-cell recruitment. Although this is in contrast to previous suggestions and findings, our experiments were carried out in an *ex vivo* setting and cannot therefore fully replicate the psoriasis microenvironment.

Further studies are needed to elucidate the relevant contribution of skin resident or circulating memory T-cells to the induction of neutrophil attracting factor production by keratinocytes. This could be addressed in animal knock out studies or by utilising specific T-cell phenotypes from psoriasis biopsies for co-culture studies. It would also be beneficial to examine the role of CD8+ T-cells in inducing production of chemotactic factors by keratinocytes, as these cells are known inducers of psoriasis pathogenesis. The co-culture approach, however, would have to be adapted to account for cytotoxicity. An important future objective should consider the evaluation of pro-inflammatory factor gene expression by conditioned keratinocytes. This should be done at different time points, as gene expression might cease at the later interaction stages.

The better understanding and possible manipulation of the migratory events and cellular interactions orchestrated by chemokines represents an attractive and novel therapeutic approach. This could allow for the development of novel therapeutics with improved treatment specificity, making them superior to the majority of the approved agents for the treatment of psoriasis. Furthermore, the concept of using migratory potential as a readout for the inflammatory response could have implications in predicting the success of certain drug treatments. For example, if treatment success is linked to a reduction of migratory potential, this could be used as a drug efficacy test prior to drug administration. Potentially, this could help develop a more patient-specific approach to modern psoriasis treatment.

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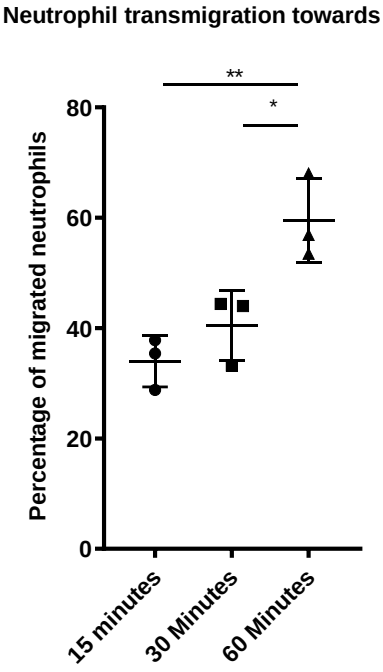
Appendices

Appendix A

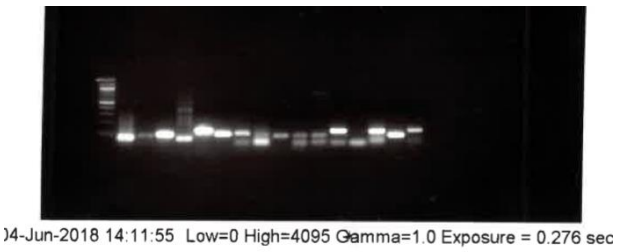


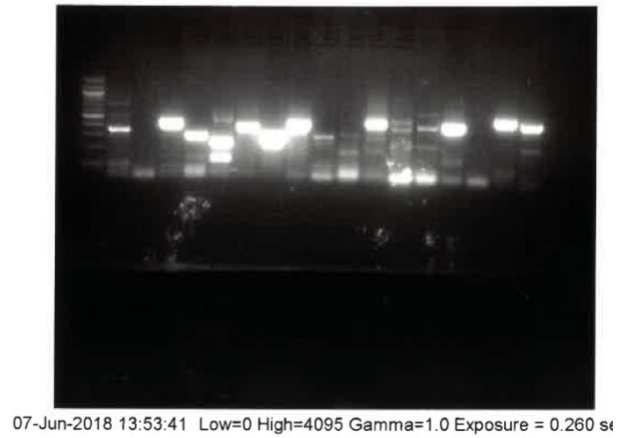
Optimisation of neutrophil migration timing towards CXCL8

Optimisation of the timing of neutrophil migration, expressed as percentage of total neutrophils, derived from psoriatic donors, in response to 50ng/mL CXCL8. Following an incubation period of 15, 30, 60 minutes in transwell chambers (6.5mm, 3μM pores), migrated cells were collected and counted. Data are presented as dot plots, with each dot representing a different biological sample, with the Mean+SD (n=3 samples) (One-way ANOVA test).

Appendix B

Primer testing via gel electrophoresis



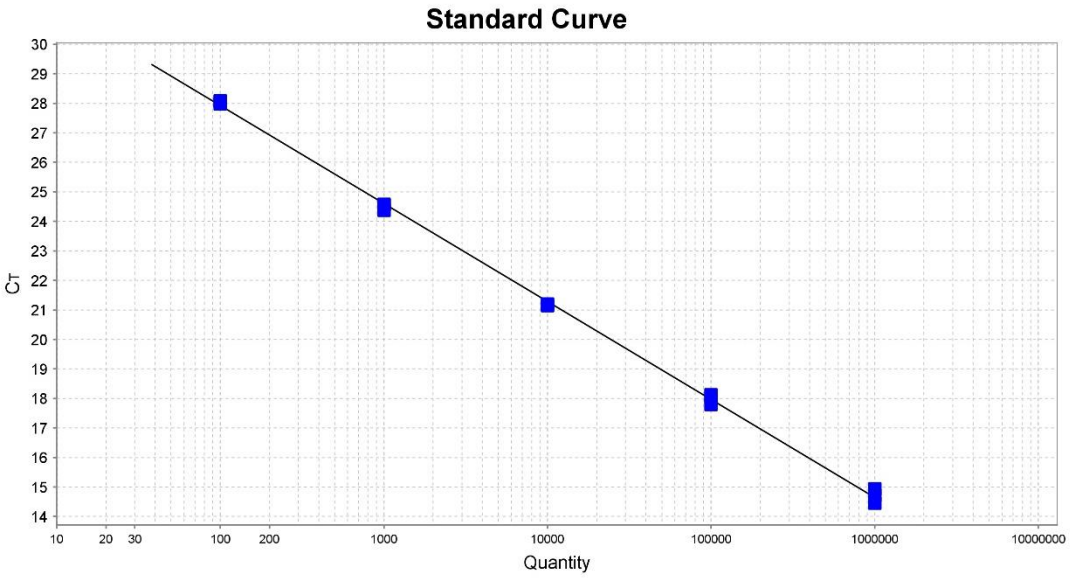


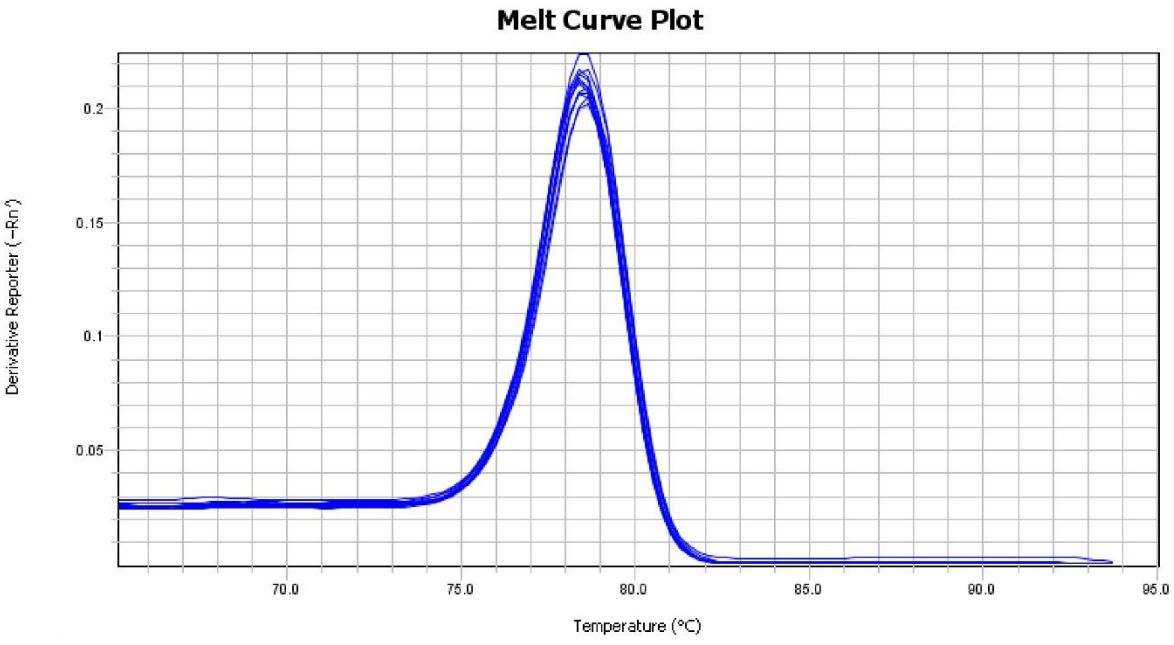
All primers purchased were tested for specificity using standard PCR with a cDNA substrate sourced from resting and activated T cells and neutrophils. The top (QPCR primers) and bottom (Standards) diagrams of agarose gel electrophoresis show the characterisation of the PCR products. From left to right on the top picture (qPCR primers): Ladder, IL-6, CXCL10, CXCL8, CCL2, IL-23a, CCL20, S100A7, LL-37, VEGF, HNP1, IL-19, CCL5, TNF, IL-17, IL-36, TATA binding protein. From left to right on the bottom picture (Standards): IL-6, CXCL10, CXCL8, CCL2, IL-23a, TATA binding protein, S100A7, LL-37, VEGF, HNP1, IL-19, CCL5, TNF, IL-17, IL-36, CCL20 version 1, CCL20 version 2

Appendix C

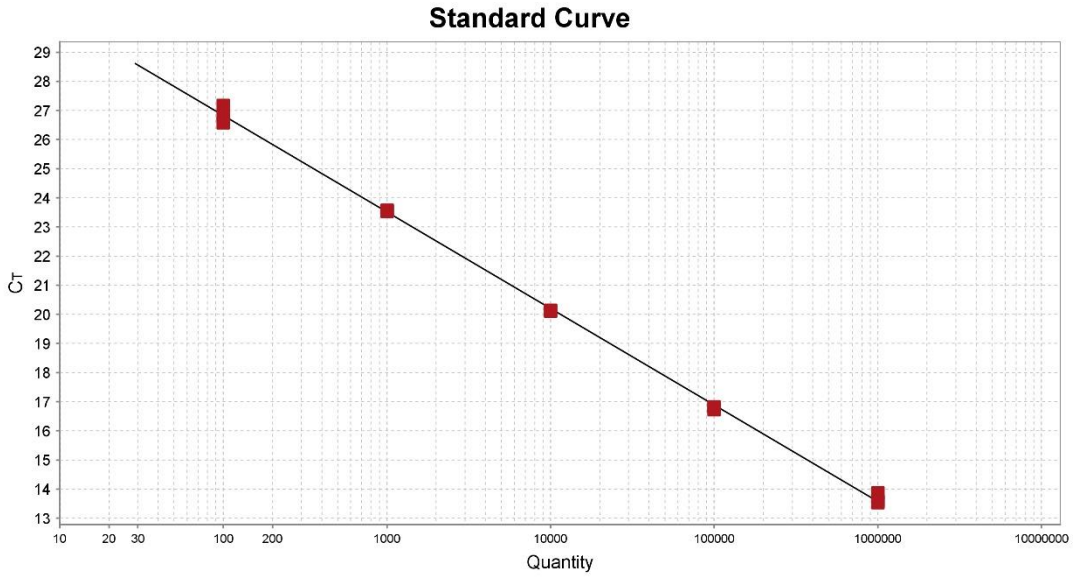
A melt curve plot and standard curves of CXCL8, TATA-binding protein, TNF, and CCL20 standard DNA templates, generated using qPCR.

CCL20





CXCL8


Standard Curve
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Quantity
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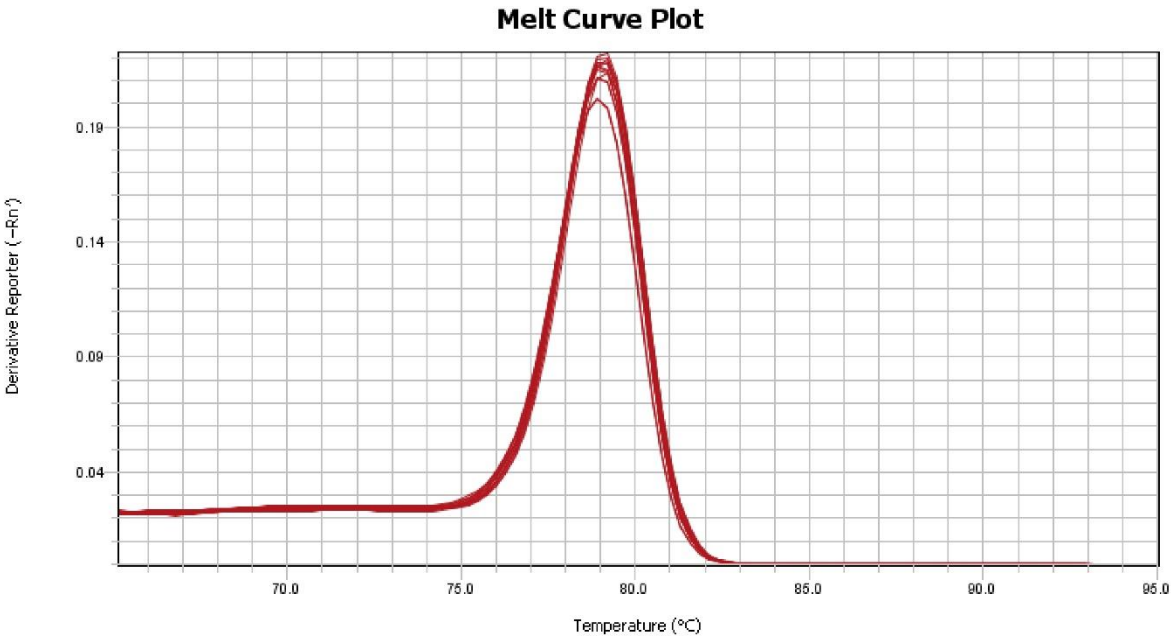
Melt Curve Plot
Derivative Reporter (-Rn')
Temperature (°C)
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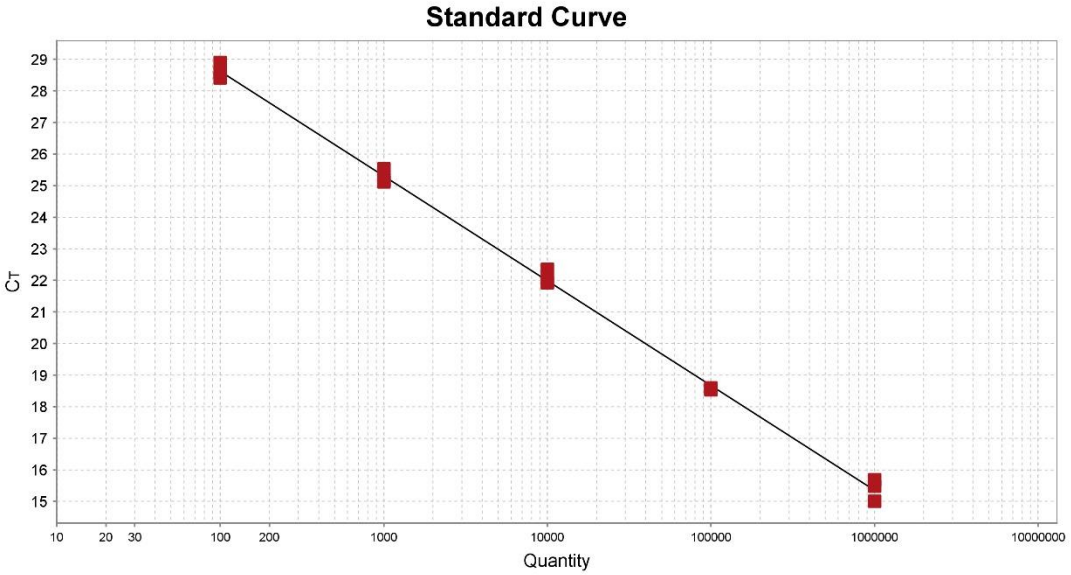
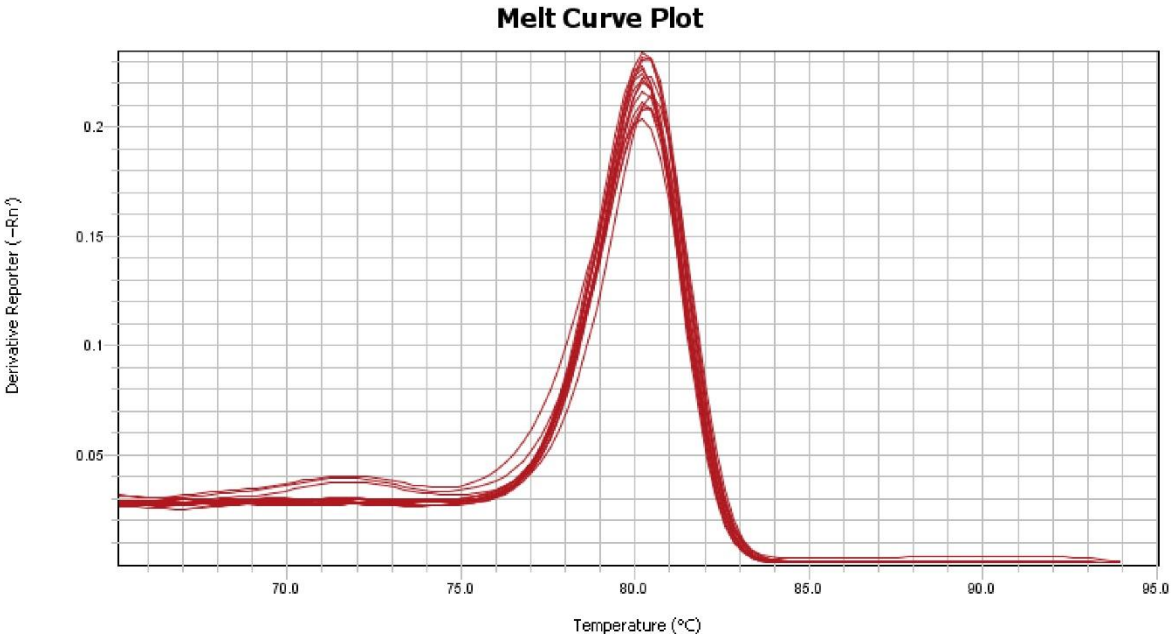
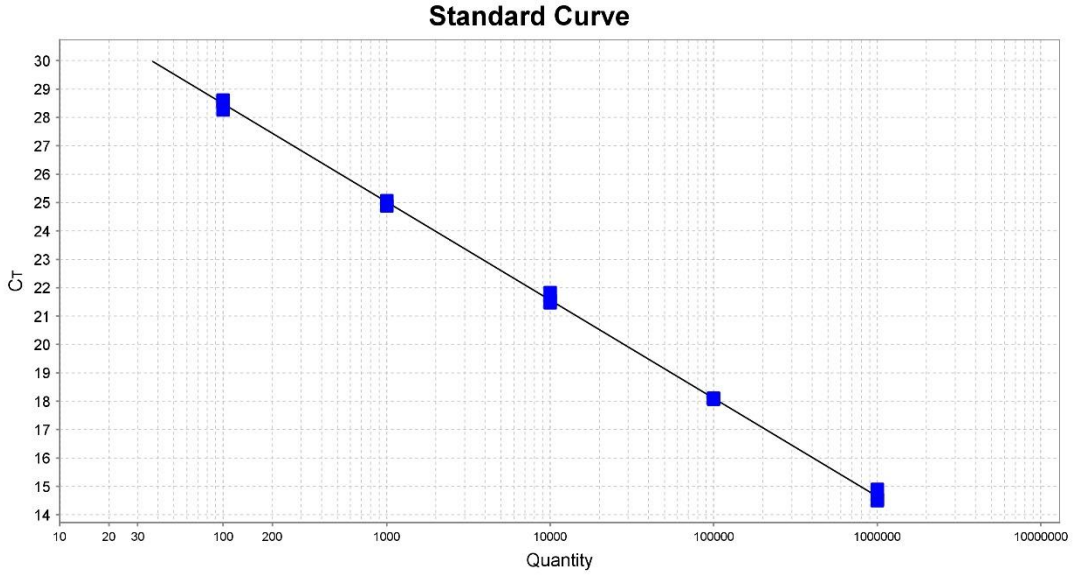
85.0
90.0
95.0

TATA-Binding Protein





TNF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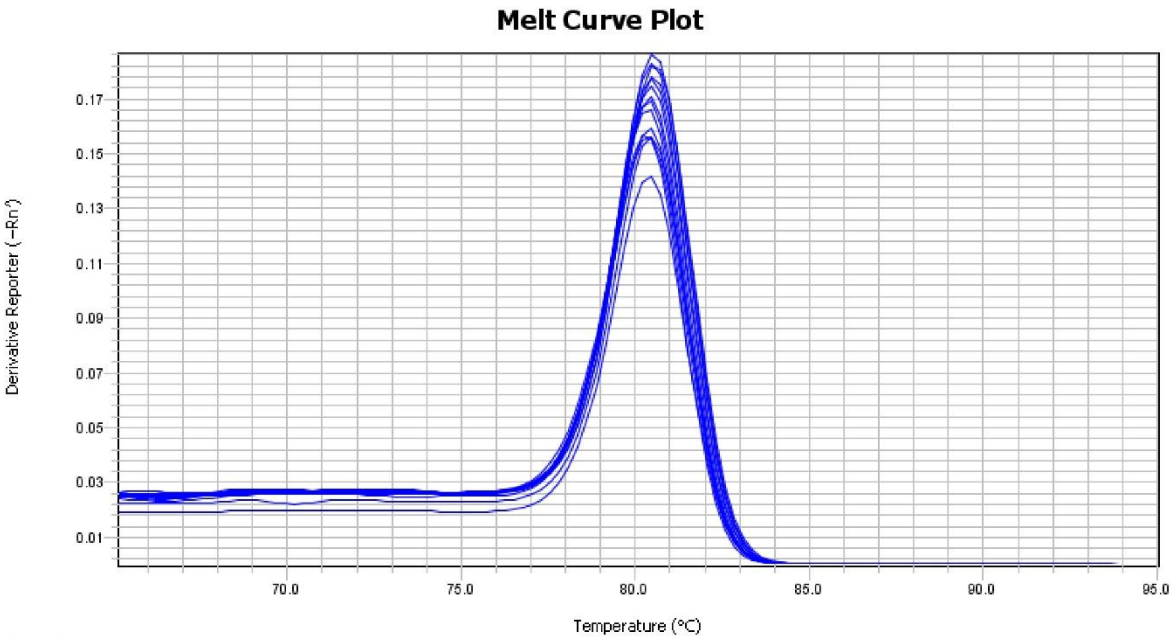
Melt Curve Plot
Derivative Reporter (-Rn')
Temperature (°C)
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Appendix D – Consent forms

Healthy controls

The Leeds Teaching Hospitals NHS
NHS Trust

PARTICIPANT INFORMATION SHEET (HEALTHY CONTROLS)

Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto) inflammatory diseases.

1. Invitation

We would like to invite you to take part in a research study. Before you decide whether you would like to take part, you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully and discuss it with your family, friends or your GP if you wish. Please take the opportunity to put any questions you may have to a qualified and experienced person. If you decide that you are happy to take part, please keep a copy of this information for future reference.

What kind of research is being done and why?

Using human tissue specimens and fluids such as urine, blood and fluid from joints in research studies is necessary to understand how the human body works normally and what changes when things go wrong. Many autoimmune and (auto)inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, vasculitis and the connective tissue diseases are associated with the presence of specific changes in either an individual's genetic makeup or their immune system. This can lead to alterations in the different components of the immune system or in the proteins that are produced by these genes. We feel these changes may be important for the development of either the disease itself, specific antibodies/ complications or that they may even predispose to or even help us predict more severe disease. We would like to perform some further research to gain a better understanding of their biology and how they may contribute to these various diseases.

Often research studies involve working with other national or international researchers in this field. Therefore, we would also like to store some of the components of your blood, such as the proteins and a sample of your DNA and urine. These samples will only be used in future studies that continue with this agreed line of research. In order to give us a permanent source of specific genes and proteins we or third parties in the UK, in Europe and/or USA may also like to generate induced pluripotent stem cells and/or make some cell-lines from specific proteins or cells from your blood in the laboratory.

Why have I been chosen?

In studies of this type we need to compare the genes, proteins and components of the immune system in people with the diseases we are interested in compared to those people of the same age who do not have the disease ("controls"). You have been identified as someone who is unlikely to have one of these conditions and we would like to store some of your blood, such as the white blood cells, proteins and sample of your DNA, and/ or a urine sample to form part of a "control" sample bank. Alternatively, you may have donated a sample for one of our previous studies and

we have already characterised some of your genes. These samples will only be used in future studies that continue with this agreed line of research.

What am I being asked to donate and what procedures are involved?

- We would ask your consent to take a small amount of blood (up to 50 mls, or 3 tablespoons teaspoons). Often at the hospital you will have a blood sample taken from you ("venepuncture") as a standard part of your clinical care. If you consent to an additional sample for research we would wherever possible take this at the same time through the same needle and therefore avoid any additional needle punctures. If it's not possible and we have to get a sample for research at a different time to your routine venepuncture for clinical blood tests it involves exactly the same process.
- Alternatively, we may ask for a urine or saliva sample or to undertake a skin swab for the above studies or in order to examine specific bacterial or viral populations that may be contributing to disease.

In all cases procedures will be carried out by a fully trained member of staff.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What are the benefits or advantages of taking part?
Research studies usually take many years to complete. You will be contributing to a bank of tissue and fluid specimens, which may help to speed up research into human disease. The results of the research overall may benefit patients with a range of diseases. In addition to contributing to generating new knowledge in medical research, it may also decrease the need to rely on testing on animals. However as the research results are about improving care and tests in the future and are not current clinical tests, the results of our experiments on your samples would not be given to you individually.

As an unconditional gift, the benefits of donating tissue specimens are humanitarian rather than personal. You will not receive any financial reward, including from the successful development of any drug or treatment, which might arise from the research and later go on to make a profit.

What are the risks to me by donating my tissues and fluids?
There are no additional health risks associated with donating specimens for research purposes if they are taken as part of a normal diagnostic procedure. If we are taking a blood sample at a different time from your routine tests, the only risks would be minor bruising. If you are a patient and anything in the procedure for obtaining your specimens were to go wrong, the normal complaint mechanisms of the NHS are open to you.

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your question. If you remain

unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

What will my samples be used for?
Your samples will be used in various research projects. This may involve large-scale analysis of the proteins present in your tissues, cells and fluids to help us understand the biology of specific diseases and look for new markers. Some samples may also be used to generate induced pluripotent stem cells. These cells can change into other types of cells depending how they are treated and will be used to study immune cell function and non-immune cell function. Samples from some individuals who consent may possibly be involved in studies examining the genetic material (DNA and RNA) and may undergo a procedure called whole genome sequencing. This could determine many or all of the features of your DNA but we are interested only in results which are relevant to your illness. No results will be given back to you individually and they will be kept confidential. However, if we find any results that may be useful for your future treatment we will notify your NHS consultant who would be able to liaise with the local NHS genetics department. The aims of the various studies will depend on the research group but include discovery of new biomarkers, new treatments or to increase our understanding of the biology of the disease.

Insurance companies, however, may ask you whether you have previously had genetic tests. Should this situation arise, we advise you to answer "no" in your insurance policy application form. This is because the genetic tests we are doing, are purely for research purposes and have no bearing whatsoever on your current or future insurance policies.

Can I withdraw my consent if I change my mind?
Yes you can if the samples or data have not yet been used. Unused data and samples would, after your notice of withdrawal, be disposed of securely and respectfully. If you change your mind and your samples or data have been used, your gift may have already contributed to new knowledge. This cannot be recalled.

If you change your mind later, or you would prefer not to approach us directly, you can write confidentially to our organisation's Research and Development Dept, who will ensure that your wishes are carried out.

Who will know I am participating in the research?
The only people who will know your identity are members of hospital staff or members of the University staff who have honorary contracts with Leeds Teaching Hospital Trust, all of whom are bound by a professional duty to protect your privacy. Once you have consented to taking part, details of your donation will be entered into a secure database within the Leeds Teaching Hospitals NHS Trust or the University of Leeds and will be accessible only to selected staff. An identification number will be assigned to you, which ensures that researchers cannot identify you personally from

your donation. This will be used in any other databases where details of your donated samples and associated information are stored.

Personal identifying information, such as your name and date of birth, will be transferred from your hospital site to the team at the University of Leeds organizing the research, to enable processing of blood samples, however your name will only appear on your blood or tissue samples until they have been separated and stored. Any samples which are shared with any third parties such as collaborators in the UK and abroad will be completely anonymised; they will be linked via unique study code to clinical information such as your diagnosis, and if applicable your relationship to other donors.

The information collected about you may also be shown to authorised people from UK Regulatory Authorities, Independent Research Ethics Committee and people authorised by the organisation responsible for ensuring that the study is carried out properly; this is to ensure that the study is carried out to the highest possible scientific standards. All will have a duty of confidentiality to you as a research participant. By signing the consent form you agree to this access for the current study and any further research that may be conducted in relation to it, even if you withdraw from the current study.

Will any of my personal information be used?
We are asking for your permission for staff to access and use information from your clinical records, including those held electronically. The information we collect will only be that which is relevant to the research and will include general information such as age, gender, medication you may be on for other illnesses, whether or not you smoke and what kind of diet you eat, as well as information more specifically about the illness we are investigating such as pathology results, results of routine blood tests and any imaging results and how you respond to different treatments. Access may start at the time you donate your samples and/or be required later e.g. to look at your clinical progress. Before your information is released to researchers, it is anonymised using identification numbers. Participants' identities will not be disclosed either to other researchers or when the results of the research are made public.

Who is funding the research?
This project is partly being funded by grants from the National Institute for Health Research, Medical Research Council, Arthritis Research UK, the Wellcome Trust, the Rheumatology Department at the Leeds Teaching Hospitals NHS Trust and the University of Leeds. Occasionally we will also receive collaborative grants or contracts from companies such as pharmaceutical or diagnostic companies. These grants allow us to recover our costs, and any funds we receive in excess of our costs are used to fund further research.

Are there any other third parties involved in the research?
Researchers may collaborate with other researchers and third parties in the UK, the EU, Switzerland and the USA. They may work in universities, hospitals or the private sector. Your tissue or other samples will not, however, be sold for profit.
Who has reviewed the research?

This study was given favourable ethical opinion for conduct in the NHS by Leeds (East) Research Ethics Committee. This committee is appointed to determine that research studies are ethical and do not impair the rights or well-being of patients. We have received approval by this committee to be able to do this research study.

Will I get feedback from the research?
Any findings resulting from the research will be published in scientific or medical journals or be presented at a scientific conference. Information will also be available on the Leeds Teaching Hospitals and Leeds University websites (e.g. www.lirmm.leeds.ac.uk). The data will be anonymous and none of the patients involved in the study will be identified in any report or publication. The results of the studies using your sample and parts thereof will not be reported back to you.

Other things to consider
Your tissue may be used for research that involves:

- Export for use in research outside the UK to the EU, Switzerland and/or USA
- Commercial research e.g. developing new tests
- Your tissue will not be used for research that involves:
    - Therapeutic/reproductive cloning (the latter remaining illegal under the Human Fertilisation and Embryology Authority 2001)
    - Research involving human embryos and embryonic stem cells
    - Research involving animal-human hybrid embryos
    - Research into termination of pregnancy or contraception

Who to contact for Further Information:-
If you would like further information please contact:

Professor Ann W Morgan, Dr Sarah Mackie, Professor Michael McDermott on 0113 206 5117

Or, you can ask the person who has provided this booklet to you. You are encouraged to ask any questions you wish, before, during or after your consultation.

Alternatively if you or your relatives have any questions about this study you may wish to contact your GP or an organisation that is independent of the hospital at which you are being treated:

If you decide you would like to take part then please read and sign the consent form. You will be given a copy of this information sheet and the consent form to keep. A copy of the consent form will be filed in your patient notes, one will be filed with the study records.

You can have more time to think this over if you are at all unsure.

Thank you for taking the time to read this information sheet and to consider this study.
Thank you for reading this patient information sheet.

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT CONSENT FORM

Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto) inflammatory diseases.

Patient ID: ..... Initials: ..... Date of Birth: .....

Patient initial each point

1. I confirm that I have read and understand the information sheet dated 2/08/2013 (version 6.0) for the above study, and have had the opportunity to ask questions. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. I agree to take part in the study. _____

2. I understand that my medical records may be looked at by authorised individuals from the Sponsor for the study, the UK Regulatory Authority or the Independent Research Ethics Committee in order to check that the study is being carried out correctly. I give permission, provided that strict confidentiality is maintained, for these bodies to have access to my medical records for the above study and any further research that may be conducted in relation to it. I also give permission for a copy of my consent form to be sent to the sponsor for the study. _____

3. I understand that even if I withdraw from the above study, the data and samples collected from me will be used in analysing the results of the study, unless I specifically withdraw consent for this. I understand that my identity will remain anonymous. _____

4. I agree for any previous samples collected on me during previous ethically approved studies to be used for the purposes of the current study. _____

5. I agree for the results from this study to be submitted to a BioBank and for this to be updated from my medical and electronic records in order to support additional research activities. _____

6. I consent to the storage including electronic, of personal information for the purposes of this study and that it may be transferred in anonymised form only to third parties in the UK, the EU, Switzerland and/or USA together with my sample or parts thereof. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication. _____

7. I agree to the samples being used to create induced pluripotent stem cells and to these cell lines being stored for future research. _____

8. I agree to have genetic tests done on samples for research purposes. _____

9. I agree for the research team to keep my contact details on record and I am happy to be contacted to discuss participation in future research they are performing in this field. _____

Name of the patient	Patient's signature and the date the patient signed the Consent form
Name of the Investigator the taking written consent	Investigator's signature and date the Investigator signed consent form

Original to be retained and filed in the site file. 1 copy to patient, 1 copy to be filed in patient's notes

Psoriasis Disease Research study consent form

The Leeds Teaching Hospitals NHS
NHS Trust

PARTICIPANT INFORMATION SHEET

Psoriasis Disease Research - Biological Substudy 1a

1. Invitation

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish.

Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

2. What is the purpose of the study?

This component of the Psoriasis DiseAse Research study and is aimed at understanding the relationship between psoriasis disease and a range of biological samples (biomarkers, serum, plasma). It is hoped that this information will improve patient care. This may help in identifying which treatments will work in which individuals or which individuals are at the greatest risk of developing disease complications.

3. Why have I been chosen?

You have been diagnosed with psoriasis and are attending the dermatology and/or rheumatology service at Chapel Allerton Hospital. We are hoping to involve as many participants as possible to improve the accuracy of our data.

4. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form to confirm that you understand what is involved when taking part in this study. If you decide to take part you are free to leave the study at any time and without giving a reason. If you withdraw, unless you object, we will still keep records relating to the treatment given to you, as this is valuable to the study. A decision to withdraw at any time, or a decision not to take part, will not affect the quality of care you receive

5. What will happen to me if I take part?

We will collect one or more blood samples (no more than 75mL in total). This will be anonymised and stored for later analysis. This will not affect your treatment.

6 What do I have to do?

To take part in this study we require a blood sample. We may ask for further samples at a later date so that comparison between samples can be made.

7. What are the alternatives for diagnosis or treatment?

This does not involve any change in your diagnosis or management. If you chose to not be involved in this study your care will not be affected.

8. What are the risks and side effects of any investigations performed when taking part?

In providing a blood sample you will be at risk of complications relating to having a blood test. This includes tenderness and bruising around the area from where the blood is taken.

If you do decide to take part in the study, you must report any problems you have to your study nurse or doctor. There is also a contact number given at the end of this information sheet for you to phone if you become worried at any time. In the unlikely event of an emergency occurring during the conduct of the study, we may contact your nominated next of kin.

9. What are other possible disadvantages and risks of taking part?

It is not anticipated that your involvement in this study would put you at any additional risk that you would not otherwise be exposed to as part of your routine care.

10. What are the possible benefits of taking part?

We cannot promise the study will help you but the information we get might help improve the treatment of people with psoriasis by identifying complications of disease earlier or by identifying which treatments will be most effective.

11. What happens when the research study stops?

Complications of psoriasis may develop over many years and as such this is a long term study being run over 5 years initially. At the conclusion of the study you will continue to be seen in the dermatology department and your treatment will continue as normal.

12. What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your question. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

13. Contact Details

Your Doctor

Name Dr Philip Laws Tel. Number: 0113 3924685

Dermatology Research office Tel. Number: 0113 3924810

14. What will happen if I don't want to carry on with the study?

If you wish to withdraw from the study at any stage please let us know and we can arrange for your records to be removed.

If you withdraw consent from further study participation, unless you object, your data and samples will remain on file and will be included in the final study analysis

15. Will my part in this study be kept confidential?

If you consent to take part in this study, the records obtained while you are in this study as well as related health records will remain strictly confidential at all times. The information will be held securely on paper and electronically within the dermatology department at Chapel Allerton Hospital under the provisions of the General Data Protection Regulation (GDPR) 2018. Your name will not be passed to anyone else outside the research team or the sponsor, who is not involved in the trial. You will be allocated a trial number, which will be used as a code to identify you on all trial forms.

Your records will be available to people authorised to work on the trial but may also need to be made available to people authorised by the Research Sponsor, which is the organisation responsible for ensuring that the study is carried out correctly. A copy of your consent form may be sent to the Research Sponsor during the course of the study. By signing the consent form you agree to this access for the current study and any further research that may be conducted in relation to it, even if you withdraw from the current study.

The information collected about you may also be shown to authorised people from the UK Regulatory Authority and Independent Ethics Committee; this is to ensure that the study is carried out to the highest possible scientific standards. All will have a duty of confidentiality to you as a research participant.

The Leeds Teaching Hospitals is the sponsor for this study. We will be using information from you in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. The Leeds Teaching Hospitals NHS Trust will keep identifiable information about you no more than 15 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw

from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information http://www.leedsth.nhs.uk/research/how-is-your-personal-information-handled-in-research/
We will keep your name and contact details confidential and will not pass this information outside the organisation. We will use this information as needed, to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Certain individuals from The Leeds Teaching Hospitals NHS Trust and regulatory organisations may look at your medical and research records to check the accuracy of the research study. The people who analyse the information will not be able to identify you and will not be able to find out your name or contact details

With your permission, your GP, and other doctors who may be treating you, maybe notified that you are taking part in this

16. Informing your General Practitioner (GP)
This sub study involves a blood sample only and as such we will not routinely inform your GP of your involvement.

17. What will happen to any samples I give?

The samples you provide will be given a trial number (anonymised) and stored for later analysis. These will initially be stored for the duration of the study (5 years) in local facilities. After completion of the study we may request that samples are stored for future use in similar studies or transferred to a tissue bank approved by regulatory bodies for storage and later use in similar research projects. These samples would be fully anonymised.

18. Will any Genetic testing be done?

There will be no genetic testing relating to this part of the study. There is a separate sub-study in which genetic tests will be done and will require a separate consent form to be completed.

19. What will happen to the results of this clinical trial?

The data collected in this study will be periodically assessed to identify patterns of disease and potential complications of disease and will usually be published in a medical journal or be presented at a scientific conference. The data will be anonymous and none of the participants involved in the trial will be identified in any report or publication.

Should you wish to see the results, or the publication, please ask your study doctor.

20. Who is organising and funding this clinical trial?

This is being organised by doctors based at Leeds Teaching Hospitals NHS Trust and is being supported by funding from grants and awards made to the department.

21. Who has reviewed the study?

This study was given favourable ethical opinion for conduct in the NHS by Yorkshire & The Humber - Leeds West Research Ethics Committee.

22. Contact for further information

You are encouraged to ask any questions you wish, before, during or after your treatment. If you have any questions about the study, please speak to your study nurse or doctor, who will be able to provide you with up to date information about the drug(s)/procedure(s) involved. If you wish to read the research on which this study is based, please ask your study nurse or doctor. If you require any further information or have any concerns while taking part in the study please contact one of the following people:

If you decide you would like to take part then please read and sign the consent form. You will be given a copy of this information sheet and the consent form to keep. A copy of the consent form will be filed in your case notes, one will be filed with the study records and one may be sent to the Research Sponsor.

You can have more time to think this over if you are at all unsure.

Thank you for taking the time to read this information sheet and to consider this study.

The Leeds Teaching Hospitals NHS
NHS Trust

PARTICIPANT CONSENT FORM

Psoriatic DiseAse Research – Biological Substudy 1a

Participant ID: …..…….. Initials: …….. Date of Birth: ……...

Participant to initial each point

1. I confirm that I have read and understand the information sheet dated 3rd Dec 2019 (version 4.0) for the above study, have had the opportunity to ask questions. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. I agree to take part in the study. ____

2. I understand that my medical records may be looked at by authorised individuals from the Sponsor for the study, the UK Regulatory Authority, Independent Ethics Committee or from the NHS Trust in order to check that the study is being carried out correctly. I give permission, provided that strict confidentiality is maintained, for these bodies to have access to my medical records for the above study and any further research that may be conducted in relation to it. I also give permission for a copy of my consent form to be sent to the Sponsor for the study. ____

3. I understand that even if I withdraw from the above study, the data and samples collected from me will be used in analysing the results of the trial, unless I specifically withdraw consent for this. These samples will be stored for the duration of the study and may be stored beyond this providing satisfactory ethical approval has been granted. I understand that my identity will remain anonymous. ____

4. I understand that my identity will remain anonymous. ____

5. I consent to the storage including electronic, of personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication. ____

6. I agree that my GP, or any other doctor treating me, may be notified of my participation in this study. ____

Name of the participant	Participant's signature	DATE the participant signed the consent form
Name of the Investigator taking written consent	Investigator's signature	DATE the Investigator signed the consent form

Original to be retained and filed in the site file. 1 copy to participant, 1 copy to be filed in patient's notes.

Rheumatoid Arthritis Disease Research study consent form

RADAR PIS/Main study v9.0: dated 11.01.18

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT INFORMATION SHEET & CONSENT FORM

Investigator: Professor Paul Emery

Study Title: RADAR: Rheumatoid Arthritis DiseAse Research

Protocol Number: RR09/9134

Study Sponsor: University of Leeds

Subject No.: _____ Initials: _____ Date of Birth: _____

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your doctor if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

You can find independent information on participating in clinical trials on the following web site: http://www.clinicaltrialsportal.co.uk/medical-volunteers-patients.html. If you do not have access to the internet, or prefer a hard copy of the information, please ask the study doctor or nurse, who will be happy to provide you with one.

1. What is the purpose of the study?

Inflammatory arthritis is a common condition and we would like to find out more about both its very early stages and how it changes and develops into a persistent, long term condition. We know that early treatment of inflammatory arthritis gives better results than if treatment is delayed. Unfortunately, it can be very difficult to catch patients in the early stage of the disease as often symptoms can be mild and can be atypical, such as initially just single joint involvement.
The University of Leeds aims to develop a comprehensive research program to investigate the clinical characteristics of inflammatory arthritis patients attending the Early Arthritis Clinic and those with more established disease, who are already on treatment and are attending our treatment monitoring clinics. This would be of great benefit in providing further insights into the disease in order to assist with future treatments.

The reason behind studying people at an early stage, when symptoms are new and may be associated with only small amounts of joint swelling, is that such individuals hold the key to understanding what makes some people develop a persistent arthritis and others go into remission. By identifying the first changes of inflammatory arthritis using the most sensitive techniques available and comparing and contrasting that with patients who have more advanced arthritis, our knowledge will be significantly advanced. This ultimately will help improve early diagnosis and tailor treatment to the individual. We therefore want to collect data on all patients with possible or probable or definite inflammatory arthritis over time, producing a database from which we can address these important research questions.

2. Why have I been chosen?

You have been invited to participate in this study because you have been identified as having possible or probable or definite inflammatory arthritis. We want to assess people like you over time to learn more about inflammatory arthritis.

3. Do I have to take part?

It is up to you to decide whether or not to take part in the study. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw from the study at any time and without giving a reason. This will not affect the standard of care you receive.

4. What will happen to me if I take part?

Your arthritis will be treated according to our usual treatment guidelines. This is NOT a study looking at new or experimental treatments and there will be little change in the care that you receive from your rheumatologist. However, your answers and findings from the routine questions asked and examinations that occur within your normal consultation with your rheumatologist will be recorded into a secure database. Results from any investigations you undergo will be recorded. Retrospective data from your clinical notes may also be recorded.

Only your initials and your unique study number and no other personal identifiers will be recorded on the database. Access to the database will be protected by password and restricted to only those involved in the study.

Patients will be asked to return for outpatient visits approximately every 3 to 6 months and then annually thereafter, unless there is a clinical need to see you more or less often. This is the same number of visits as would happen in normal clinical care.

Your doctor or nurse will carry out the following procedures which are over and above the standard clinical care you would receive if you were not taking part in the study:

- If you decide to take part, you will have the opportunity to discuss in detail what participation means and you will then need to sign the consent form as part of your initial visit for the study.
- You may also be asked to take part in one or more optional sub-studies. These include additional procedures which are over and above standard care. Each sub-study has an additional patient information sheet which outlines the procedures. Your Doctor or Nurse will discuss these with you.

At your study visits, we will capture clinical information that is part of routine care. As part of your normal hospital visit your Doctor and Nurse will:

- Ask questions about your medical history and medications you have been taking.
- Conduct routine physical examinations including blood pressure, pulse rate, height and weight.
- Take blood samples for routine arthritis tests, including fasting samples if applicable.
- Assessments will be completed regarding your current symptoms, general well-being and ability to carry out everyday tasks.
- X-rays of your hands and/or feet will be conducted at your initial visit and annually. An x-ray is a commonly used diagnostic procedure. The small dose of radiation you receive will be the same as any standard x-ray and should not be a significant health risk.
- Ultrasound (US) of your hands, feet and other joints may be conducted when clinically relevant to your care. Ultrasound is done frequently on patients in our early arthritis clinic and is standard practice in our clinic. An ultrasound scan involves placing special jelly on your joints (in this case over your hand and knee) and then a scanner, which is in the shape of a flat probe, is run over this will last approximately 30 minutes. This should not be painful or uncomfortable in any way.

- Annual questions about your cardiovascular health:
  - The amount of exercise you do normally
  - Your dietary intake (including how much fruit and vegetables you eat)
  - Any family history of premature cardiovascular disease
  - Chest pains (if you have them)

5. What do I have to do?

There should be no reason to change your current way of life if you participate in this study. As with normal clinical appointments, if you decide to take part in the study you should:

- Tell the Rheumatology doctor about any illness that you currently have or have had in the past.
- Tell your Rheumatology doctor about any other medications you are taking, as well as those supplied by your doctor.
- Keep the appointments for your study visits.
- Tell your Rheumatology doctor about any changes in your health that occurs during the study

6. What if I do not wish to take part?

All studies are always completely voluntary. If you do not wish to take part this will not affect the treatment or care you receive.

7. What are the possible disadvantages and risks of taking part?

Your care will not be greatly affected by consenting to this study. You will not require any further procedures to those that you would already undergo routinely as part of your care within the rheumatology department.

8. What are the possible benefits of taking part?

It cannot be guaranteed that you will gain personal benefit from this study: however, beneficial information may be acquired for patients who develop rheumatoid arthritis and may help us to treat these future patients better.

9. What if new information becomes available?

Sometimes during the course of a research project, new information becomes available. If this happens, your Rheumatology doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your Rheumatology doctor will make arrangements for your care to continue. If you decide to continue in the study, you will be asked to sign an updated consent form after reading a new information sheet.

Also, on receiving new information, your Rheumatology doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

10. What happens when the research study stops?

Once the study is over, your research doctor will decide whether you need to be continued to be monitored in the Rheumatology Unit or whether you can be followed by your GP.

11. Other information

All your written and computer records will be kept strictly confidential at all times. Data Protection Act regulations have been complied with to ensure confidentiality.

12. What will happen to the results of the research study?

The results from the study will be compiled on a database. These results will be analysed at various stages by Statisticians to look into markers of diagnosis, prognosis and treatment response. These results may be presented at Rheumatology meetings and published in the medical literature. All data will be fully anonymised.

13. Who has reviewed the study?

The Leeds (West) Research Ethics Committee has reviewed this study.

14. Who is funding the study?

The RADAR research programme covers several lines of investigation and is supported by a portfolio of funding including organisations and charities such as the National Institute for Health Research (NIHR) and Arthritis Research UK, and also industries such as Pfizer.

15. Contact for further information

In the event of study related questions or problems, please contact the following telephone number:

Chapel Allerton Hospital
Research Nurses officeDuring working hours

- Research room tel: (0113) 39 24729

After hours

- Ward C2 tel: (0113) 39 24202

Finally, thank you for taking the time to read the information and considering whether to take part in this study.

The Leeds Teaching Hospitals NHS
NHS Trust

CONSENT FORM -

Title of Project: Rheumatoid Arthritis DiseAse Research (RADAR)

SUBJECT INITIALS ☐☐☐ SUBJECT NO. ☐ SUBJECT DOB ☐☐☐

Name of Researcher:……

Please initial line

1. I confirm that I have read and understand the information sheet dated 11.01.18 (version 9.0) for the above study, and have had the opportunity to ask questions. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. I agree to take part in the study. _____

2. I understand that my medical records may be looked at by authorised individuals from the Sponsor for the study, the UK Regulatory Authority or the Independent Ethics Committee in order to check that the study is being carried out correctly. I give permission, provided that strict confidentiality is maintained, for these bodies to have access to my medical records for the above study and any further research that may be conducted in relation to it. I also give permission for a copy of my consent form to be sent to the Sponsor for the study. _____

3. I understand that even if I withdraw from the clinical study, the data and samples collected from me will be used in analysing the results of the study, unless I specifically withdraw consent for the laboratory study. I understand that my identity will remain anonymous. _____

4. I consent to the storage including electronic and personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication. _____

5. I agree for any previous samples collected on me during previous ethically approved studies to be used for the purposes of the current study. _____

6. I agree for the results from this study to be submitted to the Rheumatology department BioBank and for this to be updated from my medical and electronic records in order to support additional research activities. _____

7. I consent for my GP to be informed of my participation in this study _____

Name of Patient	Date	Signature
(Please print your name and date your own signature)		

Name of Person taking consent	Date	Signature
(Investigator/delegated medically – qualified sub investigator)		

Original copy – site file; 1 copy for patient; 1 copy to be kept with hospital notes